

**The contribution of T cell-derived cytokines and proteases
to chronic inflammation in the human intestine**

by

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This work is dedicated to the memory of my grandparents
Margherita, Luigi, Bianca, and Paolo

Statement of originality

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Details of collaboration

I declare that the work presented in this thesis is my own, with the exception of some of the experiments reported in Chapters 3, 4 and 6, which, upon obtaining approval from the owners of the data, have been included in this thesis and have been listed in the Note at the beginning of the Chapters.

Details of publications – Original papers

Biancheri P, Pender SL, Ammoscato F, Giuffrida P, Sampietro G, Ardizzone S, Ghanbari A, Curciarello R, Pasini A, Monteleone G, Corazza GR, Macdonald TT, Di Sabatino A. The role of interleukin 17 in Crohn's disease-associated intestinal fibrosis. *Fibrogenesis Tissue Repair* 2013;6:13.

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Abstract

This Thesis explores aspects of disease pathogenesis in Crohn's disease (CD) and ulcerative colitis (UC), investigates mechanisms of responsiveness to biologic treatment in inflammatory bowel disease (IBD), and describes the clinical and immunologic phenotype associated with a homozygous deletion in a *disintegrin and a metalloproteinase (ADAM)17* gene.

The role of interleukin (IL)-17A, IL-17E and IL-13 in human intestinal inflammation is not clear. IL-13 plays an important role in experimental colitis and in intestinal experimental fibrosis. However, contrasting observations exist on the levels and the role of IL-13 in inflamed IBD mucosa, and limited information is available on the role of IL-13 in CD intestinal fibrosis. We observed that IL-13 is not up-regulated in UC intestinal mucosa, and that it is unlikely to play a functional role in the mucosal pro-inflammatory response in the majority of patients with UC. Conversely, IL-17A is up-regulated in fibrostenosing CD intestine, and may contribute to intestinal fibrosis in CD. Our results indicate that IL-17E and IL-13 are not up-regulated in CD intestinal strictures, and are unlikely to play a role in intestinal fibrosis in CD.

A considerable proportion of IBD patients do not respond to anti-tumour necrosis factor (TNF)- α agents. Anti-TNF- α agents exert their action in inflamed tissues, rich in matrix metalloproteinase (MMP)-3 and MMP-12, which in turn can degrade immunoglobulin (Ig)G₁. We observed that MMP-3, MMP-12, and protein extracts from inflamed IBD mucosa, but not MMP-9, degrade the anti-TNF- α agents infliximab, adalimumab and etanercept, however etanercept shows a higher susceptibility than infliximab and adalimumab. We also observed that a subgroup of IBD patients who did not respond to anti-TNF- α agents have particularly high serum levels of MMP-3/MMP-12-cleaved endogenous IgG and anti-hinge autoantibodies compared to IBD patients who subsequently responded to biologic therapy.

Finally, we observed that homozygous deletion in *ADAM17* in humans is associated with a complex, neonatal-onset, multi-organ syndrome affecting mainly the skin, the intestine, and the cardiovascular system. In this condition, ADAM17 expression is down-regulated in the skin and in the duodenum, and soluble TNF- α release by peripheral blood mononuclear cells (PBMCs) is substantially impaired.

These results underline the heterogeneity characterising chronic intestinal inflammation, and may form the basis for subsequent studies with the aim to identify accurate serum biomarkers of disease progression and responsiveness to biologic therapy, and ultimately to develop effective strategies of patient stratification in IBD.

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List of abbreviations

ADAM	A disintegrin and a metalloproteinase
A/E	Attaching-and-effacing
AhR	Aryl hydrocarbon receptor
APC	Antigen-presenting cell
APMA	Aminophenylmercuric acetate
APRIL	A proliferation-inducing ligand
ASCA	Anti- <i>Saccharomyces cerevisiae</i> serum antibodies
BAFF	B cell activating-factor of the tumour necrosis factor family
CARD15	Caspase recruitment domain-containing protein 15
CAI	Clinical Activity Index
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CD	Crohn's disease
CDAI	Crohn's Disease Activity Index
CDEIS	Crohn's Disease Endoscopic Index of Severity
CEACAM	Carcinoembryonic antigen-related cell adhesion molecule
CRAMP	Cathelicidin-related antimicrobial peptide
CRC	Colo-rectal cancer
CroP	<i>Citrobacter rodentium</i> outer membrane protease
CRP	C reactive protein
CT	Computed tomography
CTLA	Cytotoxic T lymphocyte antigen
CXCL	Chemokine (C-X-C motif) ligand
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DSG	Desmoglein
DSS	Dextran sulfate sodium
EAEC	Enteraggregative <i>Escherichia coli</i>
ECM	Extracellular matrix

EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EHEC	Enterohaemorrhagic <i>Escherichia coli</i>
EIEC	Adherent-invasive <i>Escherichia coli</i>
ELISA	Enzyme-linked immunosorbent assay
EPEC	Enteropathogenic <i>Escherichia coli</i>
ESR	Erythrocyte sedimentation rate
FAE	Follicle-associated epithelium
FasL	Fas ligand
FBS	Foetal bovine serum
Fc γ R	Fc γ receptor
FcRn	Neonatal Fc receptor
GALT	Gut-associated lymphoid tissue
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GWAS	Genome-wide association studies
HLA	Human leukocyte antigen
HSP	Heat shock protein
IBD	Inflammatory bowel disease
IBDU	IBD, type unclassified
ICAM	Intercellular adhesion molecule
IEC	Intestinal epithelial cells
IEL	Intraepithelial lymphocytes
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-4R	Interleukin-4 receptor
IL-6R	Interleukin-6 receptor
IL-7R	Interleukin-7 receptor
IL-13R	Interleukin-13 receptor
IL-17R	Interleukin-17 receptor

<i>IL23R</i>	<i>Interleukin-23 receptor</i> (gene)
IL-23R	Interleukin-23 receptor (protein)
ILC	Innate lymphoid cell
ILC2	Type 2 innate lymphoid cells
ILF	Isolated lymphoid follicle
iNKT	Invariant natural killer T
IRF	Interferon regulatory factor
iRhom	inactive Rhomboid
JAK	Janus kinase
JAM	Junctional adhesion molecule
KLH	Keyhole limpet haemocyanin
LPMC	Lamina propria mononuclear cell
LPS	Lipopolysaccharide
LTi	Lymphoid tissue inducer
MAdCAM	Mucosal addressin cell adhesion molecule
MALT	Mucosa-associated lymphoid tissue
MAPK	Mitogen-activated protein kinase
MDP	Muramyl dipeptide
MDR1	Multi-drug resistance type 1
MHC	Major histocompatibility complex
MLCK	Myosin light chain kinase
MLN	Mesenteric lymph node
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MT	Transmembrane domain
MyD88	Myeloid differentiation primary response gene 88
NF	Nuclear factor
NK	Natural killer
NKT	Natural killer T
NLR	Nucleotide-binding domain and leucine-rich repeat containing receptor

NOD2	Nucleotide-binding oligomerisation domain containing 2
OR	Odds <i>ratio</i>
p-ANCA	Perinuclear anti-neutrophil cytoplasmic serum autoantibodies
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PDAI	Perianal disease activity index
PGP	Proline-glycine-proline
PI3K	Phosphatidylinositol 3-kinase
pIgA	Immunoglobulin A polymers
pIgR	Polymeric Ig receptor
PMA	Phorbol 12-myristate 13-acetate
PP	Peyer's patch
PPAR	Peroxisome proliferator-activated receptor
PRR	Pattern recognition receptor
PUFA	Polyunsaturated fatty acid
qRT	Quantitative reverse transcription
RELM β /FIZZ	Resistin-like molecule β /found in inflammatory zone
rh	Recombinant human
RIPK	Receptor-interacting protein kinase
ROR	Retinoic acid receptor-related orphan nuclear receptor
SC	Secretory component
SES-CD	Simple Endoscopic Score in Crohn's Disease
SFB	Segmented filamentous bacterium
SIg	Secretory immunoglobulin
SNP	Single nucleotide polymorphism
STAT	Signal transducer and activator of transcription
TCNB	50 mM Tris, 10 mM CaCl ₂ , 150 mM NaCl, 0.05% Brij 35
T-LPL	Lamina propria T lymphocyte
TACE	Tumour necrosis factor- α converting enzyme
T β R	Transforming growth factor- β receptor
TCR	T cell receptor

TGF	Transforming growth factor
Th	T helper
TIMP	Tissue inhibitor of metalloproteinase
TL1A	Tumour necrosis factor-like ligand 1A
TLR	Toll-like receptor
TNBS	Trinitrobenzenesulfonic acid
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor- α receptor
Treg	Regulatory T cell
TRUC	<i>Tbx21</i> ^{-/-} <i>Rag2</i> ^{-/-} ulcerative colitis
TSLP	Thymic stromal lymphopoietin
UC	Ulcerative colitis
UCEIS	Ulcerative Colitis Endoscopic Index of Severity
VCAM	Vascular cell adhesion molecule

Chapter 1: General Introduction – Hypotheses

This Thesis explores aspects of disease pathogenesis and responsiveness to treatment in chronic inflammation in the human intestine. The following introductory Chapter provides an overview of the components and functional properties of the intestinal mucosal immune system, particularly regarding the intestinal barrier, mucosal T cells, and proteases, and outlines clinical and immunologic aspects of inflammatory bowel disease (IBD). In the last part of the Introduction, the background and hypotheses of the Results Chapters are described. Subsequently, after a general description of the Methods, the individual Results Chapters are focused on the involvement of cytokines produced by mucosal T cells in mechanisms of intestinal inflammation, on the effects of proteases on biologic agents, and on the immunologic consequences of a mutation in a particular protease gene.

1.1 The intestinal mucosal immune system

1.1.1 Constituents, organisation and development

The intestinal mucosal immune system is divided into two compartments, namely inductive sites and effector sites. Inductive sites comprise the mucosa-associated lymphoid tissue (MALT), or gut-associated lymphoid tissue (GALT) in the intestine, and the mucosa-draining mesenteric lymph nodes (MLNs). Effector sites comprise the epithelium, the lamina propria and the stroma of exocrine glands (Brandtzaeg *et al.* 2004; Brandtzaeg *et al.* 2008).

GALT and induction of the mucosal immune response

GALT, which comprises Peyer's patches (PPs), caecal and colonic patches, isolated lymphoid follicles (ILFs), and the appendix, is at the forefront of interaction between the immune system, food antigens and the intestinal

microbiota, and is important in controlling the intensity of immune response (Brandtzaeg *et al.* 2013). Similar to lymph nodes, GALT contains B cell follicles, interfollicular T cell areas, efferent lymphatics and antigen-presenting cells (APCs), but has neither capsule nor afferent lymphatics. PPs contain by definition between 5 and 200 aggregated lymphoid follicles, and can be considered immune sensors in the intestine and are important inductive sites for both antigen-specific humoral and cell-mediated immunity (Cornes 1965; Jung *et al.* 2010). PPs contain multiple B cell follicles with germinal centres, where isotype switching to immunoglobulin (Ig)A occurs, surrounded by the mantle zone filled with naïve B cells (Brandtzaeg 2010; Macpherson *et al.* 2012). Caecal and colonic patches are similar to PPs. ILFs are in close proximity to the intestinal epithelium, sample antigens through M cells, and contain germinal centers; however, ILFs contain relatively few T cells and no distinct T cell zones (Eberl *et al.* 2009).

Dome-shaped PPs are covered by follicle-associated epithelium (FAE), which lacks *villi* and contains M cells, a particular type of epithelial cells that are effective in antigen uptake from the mucosal surface (Brandtzaeg *et al.* 2013). Dendritic cells (DCs) are localised underneath the FAE for capture, processing and presentation of antigens delivered by M cells, and subsequent initiation of antigen-specific immune responses (Fujihashi *et al.* 2013). Luminal antigen sampling can take place also outside inductive sites, in which case it is typically operated by lamina propria DCs, extending their dendrites between epithelial cells to reach the antigens, without compromising the integrity of the epithelial barrier (Rescigno *et al.* 2001). The interaction between antigens sampled from mucosal surfaces with naïve B and T cells can take place both in GALT and MLNs, and leads to the differentiation of memory and effector cells, which may first enter lymphatic, then peripheral blood circulation, then extravasate at mucosal effector sites. This process, named “homing”, is directed by tissue expression of vascular adhesion molecules and chemokines, and by the expression of integrins and chemokine receptors on the surface of B and T cells

(Brandtzaeg *et al.* 2008). It has been shown that DCs in PPs can educate antigen-specific T cells to acquire intestinal homing molecules, such as C-C chemokine receptor (CCR)9 and $\alpha_4\beta_7$ integrin, via vitamin A and retinoic acid (Mora *et al.* 2003; Fujihashi *et al.* 2013).

Development of the mucosal immune system

Organogenesis of PPs, MLNs and ILFs is not synchronous. It has been shown that both PPs and MLNs are programmed to start developing in the sterile foetal environment (Eberl *et al.* 2009). Organogenesis of PPs and MLNs in mice requires the expression of lymphotoxin- $\alpha_1\beta_2$ on the surface of retinoic acid receptor-related orphan nuclear receptor (ROR) γ t-expressing CD3⁻CD4⁺ interleukin (IL)-7 receptor (IL-7R)⁺ lymphoid tissue inducer (LTi) cells, of haemopoietic origin (Eberl *et al.* 2003; van de Pavert *et al.* 2010). As a consequence, LTi cells can interact with lymphotoxin- β receptor expressing stromal organiser cells, of mesenchymal lineage, and this is a pivotal event driving secondary lymphoid tissue development (van de Pavert *et al.* 2010). Whether similar processes occur in humans is difficult to determine, however a LTi cell which expresses IL-7R and RORc (the human orthologue of mouse ROR γ t), but is CD4⁻, has been described in foetal human tissue (Cupedo *et al.* 2009). In humans, PP precursors begin to form at 11 weeks of pregnancy, and at 19 weeks of gestation organised small PPs with a FAE, primary B cell follicles, T cell areas and a population of CD11c⁺ DCs below the FAE can be distinguished. Soon after birth, presumably as a result of colonisation by the intestinal microbiota, early secondary follicles in developing germinal centres appear, and PPs enlarge to become macroscopically visible (Brandtzaeg *et al.* 2008). Approximately, there are 50 PPs at the beginning of the last trimester, 100 at birth, 250 at 15 years, and then the number of PPs progressively decreases to become approximately 100 between 70 and 95 years of age (Cornes 1965).

Unlike PPs and MLNs, organogenesis of other human GALT structures, including ILFs, commences only after birth upon bacterial colonisation of the intestine (Eberl *et al.* 2009). The different timings of appearance of intestinal lymphoid tissues strongly suggest that development is programmed in the case of MLNs and PPs, and environment-induced in the case of ILFs (Eberl *et al.* 2009). In germ-free mice, ILFs do not develop, and a comparable amount of small clusters of LT α i cells, named cryptopatches, are present instead (Eberl *et al.* 2004; Bouskra *et al.* 2008). It has been suggested that cryptopatches are clusters of LT α i cells that induce the formation of ILFs during bacterial colonisation (Bouskra *et al.* 2008). The size and number of ILFs depends on the levels of intestinal bacteria, and, during early life, microbial colonisation induces substantial changes in the epithelium, with important consequences on barrier integrity and function. In particular, the intestinal microbiota exerts an important influence on the maturation of the mucosal immune system by driving the development of secondary lymphoid follicles, induction of secretory Ig (SIg)A, differentiation of innate immune cells, and shaping of T cell subsets (Hooper *et al.* 2012).

1.1.2 The intestinal epithelium

The intestinal epithelium is formed by a single layer of columnar and cuboidal intestinal epithelial cells (IECs) and a number of specialised epithelial cells, including mucus-releasing goblet cells, enteroendocrine cells, tuft cells and, in the small intestine, Paneth cells. M cells are one further type of specialised epithelial cells, which ensure controlled interaction between luminal antigens and cells of the intestinal mucosal immune system (Pickard *et al.* 2010). Furthermore, interspersed between IECs are intraepithelial lymphocytes (IELs).

With a surface of approximately 300 m², the intestinal epithelium is the largest interface between the body and the external environment, and is exposed to food components and to the luminal microbiota (Turner 2009; Barmeyer *et al.* 2015). The intestinal epithelium absorbs nutrients and water, but does so in an environment containing a high density of dietary and microbial antigens (Rescigno 2011). Hence, the integrity of the intestinal epithelium is critical for immune homeostasis and its disruption may result in uncontrolled antigen ingress into the mucosa and the subsequent development of inflammatory reactions. The impermeability of the intestinal epithelium is regulated by intercellular junctions between IECs which, from the luminal to the baso-lateral side, are represented by the tight junctions (formed mainly by claudins and *zonula occludens* proteins), the adherens junctions (formed mainly by homotypic interactions involving E-cadherin) and the desmosomes (formed by interactions between desmoglein (DSG), desmocollin, desmoplakin and keratin filaments) (Turner 2009).

IECs – Protein expression profile

IECs express a wide range of receptors for cytokines, including pro-inflammatory tumour necrosis factor (TNF)- α , IL-1 β , IFN- γ , and regulatory IL-22, as well as the common γ chain, which is required for IL-2, IL-4, IL-7, IL-9 and IL-15 signalling

(Reinecker *et al.* 1995). In particular, TNF- α , IL-1 β , IFN- γ and lipopolysaccharide (LPS) impair tight junction impermeability, thus accelerating the onset of experimental colitis (Moriez *et al.* 2005; Wang *et al.* 2005; Al-Sadi *et al.* 2008). IL-13 can alter claudin expression and composition in the tight junctions, hence promoting intestinal barrier dysfunction. In particular, IL-13 up-regulates the expression of claudin 2 in monolayers of T84 and HT-29 cells, with consequent reduction of transepithelial resistance and up-regulation of paracellular permeability (Heller *et al.* 2005; Prasad *et al.* 2005; Weber *et al.* 2010). IL-22, produced by T helper (Th)17 cells and natural killer (NK) cells, plays an important role in intestinal wound healing by inducing the activation of signal transducer and activator of transcription (STAT)3 in IECs and consequently orchestrating a pro-regenerative, wound healing programme (Pickert *et al.* 2009). Moreover, IL-17A enhances tight junction function between polarised IECs (Kinugasa *et al.* 2000).

IECs constitutively express major histocompatibility complex (MHC) class I molecules and, upon stimulation with pro-inflammatory molecules such as interferon (IFN)- γ , murine IECs can also express MHC class II, particularly in the small intestine (Thelemann *et al.* 2014). Moreover, on the surface of IECs there are non-classical MHC class I molecules, including CD1d and human leukocyte antigen (HLA)-E (Perera *et al.* 2007). IECs also express receptors called pattern recognition receptors (PRRs), that recognise structures common to both pathogens and commensal microorganisms, such as LPS, peptidoglycan, flagellin and lipoteichoic acid (Gewirtz *et al.* 2001; Lavelle *et al.* 2010; Fukata *et al.* 2013). In particular, PRRs include membrane-associated toll-like receptors (TLRs) and intracellular nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs) (Lavelle *et al.* 2010). After ligand binding, intracellular signalling of most TLRs, except for TLR3, is dependent on myeloid differentiation primary response gene 88 (MyD88) (Fukata *et al.* 2013). Both MyD88 signalling and muramyl dipeptide (MDP)-sensing by the nucleotide-binding oligomerisation domain containing 2 (NOD2) NLR induce nuclear factor

(NF)- κ B activation, whereas MyD88-independent TLR3 and TLR5 activation leads to caspase activation and type I IFN production (Baumgart *et al.* 2007a; Fukata *et al.* 2013). While IECs express only TLR2, TLR3, TLR4, TLR5 and TLR9, professional APCs express the full spectrum of PRRs (Iwasaki *et al.* 2003; Fukata *et al.* 2013). NOD2 is mainly expressed by Paneth cells (Lala *et al.* 2003).

Particularly in response to interaction with luminal pathogens, IECs can release a variety of cytokines and chemokines with chemoattracting effects on neutrophils, such as IL-8, also known as chemokine (C-X-C motif) ligand (CXCL)8, on macrophages and DCs, such as C-C chemokine ligand (CCL)2, and on $\alpha_4\beta_7$ T cells, such as CCL25 (Kagnoff 2014). IEC expression of the carcinoembryonic antigen-related cell adhesion molecule (CEACAM)1 and CEACAM20, two Ig superfamily members which are important in the epithelial-lymphoid cell cross-talk process, is regulated by the intestinal commensal microbiota (Kitamura *et al.* 2015). At the steady state, IECs release regulatory molecules, including transforming growth factor (TGF)- β , retinoic acid and thymic stromal lymphopoietin (TSLP), which have been shown to be essential in driving the development of CD103⁺ DCs in mice (Iliev *et al.* 2009). Moreover, epithelial-derived TSLP plays an important role in immunologic tolerance by inhibiting IL-12 release by DCs in response to bacteria (Rescigno *et al.* 2009).

Specialised epithelial cells in the intestine

Goblet cells produce the mucin glycoproteins forming the mucus layers covering the epithelium (Birchenough *et al.* 2015). This provides both anchorage and nutrients to the intestinal microbiota, however it also minimises and regulates direct interaction of bacteria with the intestinal mucosa (Rescigno 2011). Tuft cells have been identified in the human intestine, however their function is still largely unknown (Gerbe *et al.* 2012). In the small intestine, an important action against bacterial invasion across the epithelium is provided by Paneth cells, specialised cells at the base of the crypts of the small intestine which produce

anti-microbial peptides such as defensins and lysozyme (Bevins *et al.* 2011). The mediators released by Paneth cells play an important role in shaping the composition of the intestinal microbiota, in controlling microbial growth, and in protecting against pathogens (Lai *et al.* 2009; Salzman *et al.* 2013). Enteroendocrine cells, which comprise at least 14 different cytotypes depending on their specific secretion profile, are considered chemosensors of the luminal intestinal content (Gribble *et al.* 2016). Serotonin-producing cells, also known as enterochromaffin cells, are the predominant enteroendocrine cell type and are situated among crypt and villous enterocytes throughout the entire intestinal mucosa, with maximum distribution in the small bowel (Sjölund *et al.* 1983). Serotonin exerts important immunologic effects, including stimulation of T cell recruitment and proliferation, DC activation, and pro-inflammatory cytokine production (Shajib *et al.* 2015).

Development of the intestinal epithelium

All throughout the intestine, the epithelium is continuously renewed every 4-5 days by stem cells arising from the crypts of Lieberkühn. Stem cells localised at the base of the crypts give rise to the three cell types that move toward the luminal side during their differentiation (IECs, enteroendocrine cells, and goblet cells), and, in the small intestine, to the only cell type that stays at the base of the crypt, Paneth cells (Mowat *et al.* 2014). Crypt base columnar cells were confirmed to be long-lived, multipotent stem cells, characterised by the expression of the R-spondin receptor Lgr5, which enhances Wnt signalling strength (Barker *et al.* 2007; Koo *et al.* 2014). Paneth cells constitute the ideal niche for Lgr5⁺ stem cells in intestinal crypts by expressing epidermal growth factor (EGF), TGF- α and Wnt3, all essential signals for stem cells maintenance in culture (Sato *et al.* 2011).

The Wnt pathway is the major driver of epithelial cell renewal, and Wnt-mediated activation of the bipartite transcription factor β -catenin/TCF is the central event in

establishing migratory pathways of cells in development, as well as in maintaining cellular boundaries in the intestinal epithelium (Batlle *et al.* 2002; Koo *et al.* 2014). Also Notch ligands are essential for the homeostasis of intestinal stem cells, and Notch signalling promotes the preferential development of IECs and inhibition of secretory cell lineage differentiation (Koo *et al.* 2009). Conversely, inhibition of Notch signalling leads to the conversion of all epithelial cells into goblet cells (van Es *et al.* 2005). Neurogenin 3 promotes the development of a bipotential secretory progenitor to enteroendocrine cell rather than goblet cell fate (López-Díaz *et al.* 2007). Paneth cells appear and begin to express α -defensins in the first trimester of gestation in humans and, after birth, the gene products of murine Paneth cells and the intestinal microbiota exert a reciprocal influence on each other. Wnt signalling is required for Paneth cell maturation and expression of anti-microbial peptides (McGuckin *et al.* 2013). It has been shown that crypt base columnar stem cells can be grown into budding cysts containing crypt- and villus-like domains, and harboring all 4 common lineages (IECs, enteroendocrine cells, goblet cells and Paneth cells), and these epithelial cysts can be expanded in culture over years (Sato *et al.* 2009). These structures, called intestinal organoids, can also be established from human Lgr5⁺ cells (Jung *et al.* 2011).

IELs – Phenotype, functions and development

IELs can be divided into “conventional” or “type A” IELs, which express a T cell receptor (TCR) $\alpha\beta$ in addition to the co-receptors CD8 $\alpha\beta$ or, less frequently, CD4, and are thought to derive from naïve T cells that have been activated in secondary lymph nodes; and “unconventional” or “type B” IELs, which lack CD8 $\alpha\beta$ and CD4, express either a TCR $\gamma\delta$ or a TCR $\alpha\beta$, and typically express the activation marker CD8 $\alpha\alpha$, thought to derive from agonist-selected autoreactive thymocytes (Mowat *et al.* 2014).

IELs share functional and phenotypical characteristics that distinguish them from lymphocytes present at other sites. In particular, IELs are almost exclusively T cells, and comprise a high proportion of $\gamma\delta$ T cells. IELs are antigen-experienced, however they do not express the activation marker CD25. IELs are largely cytotoxic and display constitutive cytolytic activity driven by various pathways, including serine proteases, perforin release, and Fas-Fas ligand (FasL)-mediated programmed cell death, but they release only small amounts of cytokines (Cheroutre *et al.* 2013). $CD8\alpha\beta^+TCR\alpha\beta^+$ IELs, which represent the majority of IELs in the small intestine, can be rapidly activated and provide immediate cytotoxic function. $CD4^+TCR\alpha\beta^+$ IELs include the classical Th1, Th2, Th17 and regulatory T cell (Treg) subsets. Treg IELs are particularly enriched in the small intestine, and can suppress other IEL subtypes (Cheroutre *et al.* 2013).

$\gamma\delta$ IELs play an important role in preserving epithelial integrity, as suggested by the observations that $\gamma\delta$ IEL-deficient mice have a defective expression of claudin 3 and *zonula occludens-1* in tight junction complexes and are more susceptible to epithelial transmigration of *Salmonella typhimurium* (Dalton *et al.* 2006), and are more susceptible to dextran sulfate sodium (DSS)-induced colitis due to lack of keratinocyte growth factor (Chen *et al.* 2002). Moreover, $\gamma\delta$ IELs migrate close to IECs in direct contact with bacteria and limit the possible penetration of luminal microorganisms by releasing anti-microbial peptides (Ismail *et al.* 2009; Edelblum *et al.* 2015). It has been shown that, upon exposure to *Salmonella* or to commensals, IECs release IL-23, which in turn stimulates $\gamma\delta$ IELs to produce IL-22, and this latter triggers the synthesis of angiogenin 4 by Paneth cells (Walker *et al.* 2013). Among unconventional IELs, one subset has been identified which displays a repertoire of TCRs with a pattern of MHC restriction that does not overlap with that of $CD4^+$ or $CD8\alpha\beta^+$ T cells, indicating that these IELs sense antigens that are not recognised by conventional T cell subsets (Mayans *et al.* 2014).

Differently from the steady state, under inflammatory conditions, IELs may disrupt the epithelial integrity, and the close proximity of IELs with IECs may become a detrimental factor. Indeed, IELs can also produce IFN- γ and TNF- α , and, in active coeliac disease, NKG2D receptors expressed by IELs may trigger potent cytotoxicity upon binding MHC class I polypeptide-related sequence (MIC)A on IECs (Hue *et al.* 2004).

In the human intestinal epithelium, infiltration of T cells occurs from 12 to 14 weeks of gestation, then IELs continue to rise. IEL distribution depends partly on age, species and environmental conditions. Germ-free mice have a markedly reduced number of IELs, and conventional IELs are especially depleted (Round *et al.* 2009). After birth, the increase in TCR $\alpha\beta$ IELs is not seen in germ-free conditions, indicating that their accumulation depends on the intestinal microbiota. Neonatal or foetal thymus grafting studies have demonstrated that all IELs are thymus-derived, but further maturation occurs in the intestine (McGuckin *et al.* 2013).

1.1.3 The intestinal barrier

The intestinal barrier is formed by the intestinal epithelium, by the mucus system, and by anti-microbial peptides released by IECs and Paneth cells (McGuckin *et al.* 2013). The tight junctions between IECs are another important component of the intestinal barrier, as they are rate-limiting for paracellular absorption of luminal contents (Watson 2015).

The intestinal barrier protects against physical, chemical and biological insults, is selectively permeable at homeostasis, prevents the direct contact between the luminal contents and the mucosal immune system, and regulates the interactions between microbial and dietary antigens and the different cell types populating the intestinal mucosa (Turner 2009). Although the large absorptive area of the intestinal epithelium could favour the persistence of microorganisms, the low bacterial concentration in the small intestine suggests that there are efficient mechanisms in place to prevent extensive bacterial colonisation of the epithelium. In particular, the physical separation and the tightly regulated interaction between the intestinal microorganisms and the epithelial surface, ensured by the intestinal barrier, have emerged as important factors for maintaining host-intestinal microbiota homeostasis (Johansson *et al.* 2011).

Tight junction and paracellular flux regulation

There are at least two distinct pathways of paracellular flux, the so-called “leak pathway” and “pore pathway”, that depend on the regulation of tight junction protein expression by different cytokines (Turner *et al.* 2014). The main determinant of paracellular permeability is the expression of claudin family members and occludin within the tight junctions. In particular, it was originally observed in a murine model of T cell-mediated acute diarrhoea that TNF- α activates myosin light chain kinase (MLCK), which in turn promotes endocytosis of the tight junction protein occludin within IECs, thereby enhancing the

paracellular permeability via the leak pathway (Clayburgh *et al.* 2005). On the other hand, IL-13 increases paracellular permeability via the pore pathway in intestinal epithelial monolayers *in vitro* by up-regulating claudin 2 expression within the tight junctions (Weber *et al.* 2010).

Goblet cells and the intestinal mucus system

Mucus is a highly hydrated viscous secretion released by goblet cells and permeable to macromolecules, and its main components are polymeric mucin glycoproteins. Goblet cells store large amounts of mucin granules which can be rapidly released in response to microbial or inflammatory signals (Birchenough *et al.* 2015).

The main constituent of intestinal mucus is the MUC2 mucin, which needs to be appropriately folded in the endoplasmic reticulum before release, and is present in a polymeric form, resulting in the formation of enormous net-like polymeric sheets (Ambort *et al.* 2012). Other typical mucus components are FCGBP, CLCA1, ZG16, and AGR2 (Birchenough *et al.* 2015). It has been shown that mutated MUC2 accumulates into the endoplasmic reticulum as a result of misfolding, and this process leads to the development of experimental colitis (Heazlewood *et al.* 2008). The regulatory cytokine IL-10 can reduce protein misfolding and endoplasmic reticulum stress and promotes intestinal mucin production (Hasnain *et al.* 2013). MUC2 is synthesised as a transmembrane protein, and in the small intestine it is cleaved by meprin β , which, in turn, is anchored to IEC membrane and needs to be cleaved by bacteria in order to be able to access MUC2. This is in keeping with the observation that, while in normal conditions mucus can be removed easily from the surface of the small intestine, in germ-free mice MUC2 mucin remains anchored to the goblet cells (Schütte *et al.* 2014). Moreover, germ-free mice have a decreased number of goblet cells and a reduced size of goblet cell granules, together with a

paradoxically thicker mucus layer, possibly due to reduced mucin degradation (Kandori *et al.* 1996; McGuckin *et al.* 2013).

While in the small intestine there is a single layer of mucus, in the colon the mucus system is composed of an outer, looser layer, which can be easily removed and is highly populated by microorganisms, and of a dense inner layer, which is attached to goblet cells and is largely void of bacteria (Johansson *et al.* 2008; Ermund *et al.* 2013). The pore size of the inner mucus layer hampers penetration of bacteria and luminal content, and endogenous proteases are responsible for the conversion of the inner layer into the outer one (Johansson *et al.* 2008). It has been shown that the outer mucus layer hosts a distinct microbial niche, including bacteria without specialised mucolytic capacity (Li *et al.* 2015). Mucus is continuously renewed by goblet cells, and this pushes microorganisms away from the epithelial surface toward the intestinal lumen. Moreover, without the mucus the anti-microbial peptides released by Paneth cells and IECs would be quickly diluted in the luminal content and would not be able to maintain microorganisms away from the epithelium (McGuckin *et al.* 2011).

In addition to structural mucus components, goblet cells also release resistin-like molecule β /found in inflammatory zone (RELM β /FIZZ)2, which is also induced by IL-13 and plays an important effector role in counteracting nematode infections (Artis *et al.* 2003). Parasitic helminth infections trigger marked goblet cell hyperplasia and mucus hypersecretion via a Th2 response. In particular, IL-13 has been shown to directly induce goblet cell hyperplasia and release of RELM β /FIZZ2 via STAT6 signalling (Oeser *et al.* 2015). In addition, both IL-9 and IL-17E, also known as IL-25, promote goblet cell hyperplasia and mucus hypersecretion via IL-13-dependent pathways (Steenwinckel *et al.* 2009). Finally, IL-22-deficient mice show that goblet cell hyperplasia observed in response to parasitic infection is defective despite high levels of IL-4 and IL-13 (Turner *et al.* 2013).

Intestinal anti-microbial peptides

Intestinal anti-microbial peptides may be expressed constitutively or may be induced by exposure to microbial antigens, are typically activated by proteolysis, and in humans include defensins, cathelicidin LL-37, lysozyme C, phospholipases, and C-type lectins (Bevins *et al.* 2011).

Defensins comprise α - and β -defensins, are major constituents of Paneth cells granules, and are present at high concentration at the point of release. Human Paneth cell α -defensins comprise human α -defensin 5 and human α -defensin 6, and are expressed constitutively in the small intestine, whereas human β -defensins, which are expressed ubiquitously by several different cell types, including colonic IECs, are mostly inducible (Bevins *et al.* 2011). In addition to their disruptive effect on the microbial wall, defensins can exert other actions, as displayed by the chemoattractant activity of human β -defensin 1 and human β -defensin 2 for cells expressing CCR6 (Yang *et al.* 1999). While pro- α -defensins in mouse small intestine are activated intracellularly by matrix metalloproteinase (MMP)-7 cleavage, human Paneth cells store unprocessed pro- α -defensin which is activated after secretion by trypsin (Ghosh *et al.* 2002). It has been shown that mouse pro- α -defensin can also be activated in the intestinal lumen by host and microbial proteases (Mastroianni *et al.* 2012).

Human cathelicidin LL-37 is expressed by neutrophils and IECs mainly in the small intestine, is activated by proteinase 3, prevents LPS from binding TLR4, and exerts a variety of immune effects, including chemotactic action on neutrophils and stimulation of the inflammasome activation (Vandamme *et al.* 2012; Kahlenberg *et al.* 2013). Lysozyme C, which can specifically hydrolyse peptidoglycan, and phospholipases, which degrade both phosphatidylethanolamine and phosphatidylglycerol in the bacterial wall, are expressed mainly by Paneth cells and macrophages (Bevins *et al.* 2011). The main lectin in human Paneth cells is Reg3 α , which is under the control of MyD88

and, after activation by trypsin, can bind to peptidoglycan and is bactericidal against Gram-positive species (Salzman *et al.* 2013). A reduction of the intestinal microbiota following antibiotic treatment down-regulates IEC expression of Reg3 γ , the mouse orthologue of Reg3 α (Brandl *et al.* 2008).

Th17-associated cytokines, such as IL-17A, IL-17F and IL-22 enhance the production of the anti-microbial molecules lipocalin-2 and calprotectin by IECs, and both IL-17A and IL-17F stimulate intestinal production of β -defensins (Aujla *et al.* 2008; Ishigame *et al.* 2009; Raffatellu *et al.* 2009). These mechanisms potentially prevent bacterial dissemination from the intestinal mucosa, as suggested by the increased translocation of *S. typhimurium* to MLNs and spleen in mice lacking IL-17 receptor (IL-17R)A (Raffatellu *et al.* 2008). Interestingly, IL-13, but not IFN- γ , has been shown to induce Paneth cell degranulation and release of anti-microbial peptides, suggesting that IL-13 may contribute to host defense and host-microbial homeostasis (Stockinger *et al.* 2014).

Anti-microbial peptides play an important role in defense from pathogens, as exemplified by the observation that transgenic mice expressing human α -defensin 5 or human α -defensin 6 show enhanced survival upon challenge with *S. typhimurium* via the formation of nanonets, which bind to the surface proteins and entangle the bacteria (Chu *et al.* 2012; Salzman *et al.* 2013). Anti-microbial peptides exert an important influence on the composition of the intestinal microbiota, as shown by the observations of SFB depletion in the small intestine of transgenic mice expressing human α -defensin 5, and of increased bacterial colonisation of the epithelial surface, with consequent humoral and Th1 response activation, in Reg3 γ -deficient mice (Salzman *et al.* 2010; Vaishnava *et al.* 2011). Moreover, in the intestinal microbiota of *MMP-7*^{-/-} mice, which lack mature α -defensins, the relative abundance of *Firmicutes* is increased and *Bacteroidetes* are depleted, whereas transgenic mice expressing human α -defensin 5 show the reciprocal change in intestinal microbiota composition (Salzman *et al.* 2010).

1.1.4 T cell populations in the intestinal lamina propria during homoeostasis

In the normal intestine, T cells constitute one third of the cells present in the lamina propria and in the GALT, and TCR $\alpha\beta$ T cells are predominant (MacDonald *et al.* 2011). Unlike in the epithelium, in the lamina propria CD4⁺ and CD8⁺ T cells are present at a *ratio* of approximately 2:1, and are thought to derive mainly from T cells that have been primed in secondary lymphoid organs (Mowat *et al.* 2014). The number and activity of lamina propria T lymphocytes (T-LPLs) is regulated in order to ensure that intestinal homoeostasis is the default pathway and that the onset of spontaneous inflammation is prevented (Maynard *et al.* 2009). The various T cell phenotypes, including Tregs, Th1, Th2 and Th17 cells, are differentially represented in the intestinal lamina propria during homoeostasis.

The expression of $\alpha_4\beta_7$ integrin on the cell surface provides T-LPLs with the ability to bind to mucosal addressin cell adhesion molecule (MAdCAM)-1, expressed on endothelial cells of the lamina propria microvasculature (MacDonald *et al.* 2013). The vitamin A metabolite all-trans retinoic acid drives intestinal homing of T cells present in PPs by inducing $\alpha_4\beta_7$ integrin and CCR9 expression on their cell surface (Iwata *et al.* 2004).

Intestinal lamina propria Tregs – Phenotype and differentiation

The normal lamina propria and the GALT are home to a relatively high number of Tregs (Maynard *et al.* 2009). Tregs are CD4⁺ T cells characterised by the expression of Foxp3, and can be defined as T cells able to suppress naïve T cell proliferation both *in vitro* and *in vivo* (O'Garra *et al.* 2004; Izcue *et al.* 2009). Two major Treg populations have been described, which have been designated as naturally occurring and induced Tregs (O'Garra *et al.* 2004; Barnes *et al.* 2009). Naturally occurring Tregs originate in the thymus, whereas naïve CD4⁺ T cells

can differentiate into induced Tregs when activated by TCR stimulation in the presence of TGF- β and IL-2 (Chen *et al.* 2003; Barnes *et al.* 2009). TGF- β plays an important role in the differentiation of naïve T cells into Tregs. Mice lacking the binding site for Smad3 on the *Foxp3 locus*, with consequently impaired TGF- β signalling, have a decreased number of Tregs selectively in the intestine (Schlenger *et al.* 2012). Treg differentiation is promoted by the release of TGF- β and retinoic acid by CD103⁺ DCs (Mangan *et al.* 2006; Coombes *et al.* 2007; Mucida *et al.* 2007; Sun *et al.* 2007; Li *et al.* 2008). These latter, in turn, develop upon the action of epithelium-derived TGF- β , retinoic acid and TSLP (Rescigno *et al.* 2009), and have the ability to release TGF- β upon *in vitro* stimulation with *Lactobacillus paracasei* and with *Bifidobacterium breve* culture supernatants (Bermudez-Brito *et al.* 2012; Bermudez-Brito *et al.* 2013).

Effector T-LPLs – Phenotype and differentiation

A substantial proportion of CD4⁺ T-LPLs at the steady state have the phenotype of effector-memory T cells, being CD45RO⁺, CD62^{lo}, CD69^{hi}, CD25⁺, Fas⁺ and FasL⁺ (MacDonald *et al.* 2013). Effector T-LPLs can be classified into Th cell types according to their expression of specific transcription factors and cytokines.

Th1 cells are characterised by the expression of the transcription factor T-bet and the release of IFN- γ (Neurath *et al.* 2002). IL-12 is an important factor driving the development of Th1 cells, however it is mainly expressed by DCs in peripheral lymphoid tissue (Hue *et al.* 2006). IL-23 can induce IFN- γ release by memory CD4⁺ T cells, hence it has been suggested that during homeostasis lamina propria Th1 cells are induced by an IL-23-dependent pathway or migrate to the lamina propria following induction in the periphery (Maynard *et al.* 2009).

Th2 cells are characterised by the expression of the transcription factor GATA3 and the release of IL-4, IL-5 and IL-13 (Neurath *et al.* 2002). Epithelium-derived IL-17E, also known as IL-25, is a major driver of Th2 differentiation (Fort *et al.*

2001). The main mechanism driving Th2 cell development is IL-4-mediated STAT6 activation, however Th2 cell differentiation may also occur in the absence of the IL-4/STAT6 pathway (Zhu 2015).

Th17 cells are characterised by the expression of IL-23 receptor (IL-23R), CCR6 and the transcription factor ROR γ , and by the release of IL-17A, IL-17F, IL-21 and IL-22 (Zhou *et al.* 2007; Korn *et al.* 2009). Th17 cells are induced by a combination of IL-6 and TGF- β , and their expansion is promoted by IL-23 (Zhou *et al.* 2007). IL-21 produced by Th17 cells, in turn, stimulates their expression of the IL-23R, further expanding this cell subtype by a positive autoregulatory feedback loop (Sarrazin *et al.* 2010).

One further type of effector T cells has been reported, namely Th9 cells, which are characterised by the production of IL-9 and can be induced by antigenic stimulation of naïve T cells in the presence of TGF- β and IL-4 (Dardalhon *et al.* 2008), however their presence and function in the intestinal lamina propria at homeostasis is currently unclear.

Finally, natural killer T (NKT) cells are non-conventional T cells which express CD161 and recognise endogenous and/or exogenous glycolipid antigens when presented by the MHC class I-like molecule CD1d (Middendorp *et al.* 2009). NKT cells may express an invariant TCR α chain which pairs with multiple TCR β chains and responds to α -galactosylceramide, hence these are called invariant NKT (iNKT) cells. Alternatively, NKT cells may express a diverse set of TCR α chains, responding to a less well characterised group of lipid antigens, and are therefore named non-invariant NKT cells (Middendorp *et al.* 2009). NKT cells are present in the lamina propria at homeostasis, however their precise function in this condition is unclear (O'Keeffe *et al.* 2004).

Functional properties of T-LPLs

In the normal intestinal lamina propria, Tregs and effector T-LPLs are in close proximity to each other, suggesting that Tregs may exert a dominant suppressive action on effector T-LPL development (Maynard *et al.* 2009). Tregs exert their function by producing the anti-inflammatory cytokines IL-10 and TGF- β , and by preventing both the activation and the effector function of T-LPLs that have escaped other mechanisms of tolerance (Valencia *et al.* 2006).

In the intestinal lamina propria, Th1 cells are essential in the elimination of intracellular pathogens and viruses, whereas Th2 cells are protective against parasites (Neurath *et al.* 2002). Th1 cells are normally present in the intestinal lamina propria, whereas Th2 cells are rare or absent in the normal uninfected intestine, and this is likely to reflect the relative scarcity of parasites in the intestinal microbiota of humans living in countries with good hygienic conditions (Maynard *et al.* 2009). Th17 cells are well represented in the normal intestinal lamina propria, where they promote IL-17A-mediated neutrophil recruitment and play an important role in defense against extracellular bacteria and *fungi* (Korn *et al.* 2009). Moreover, Th17-associated cytokines, such as IL-17A, IL-17F and IL-22, play an important role in mucosal immune responses to bacteria and favour epithelial barrier restitution and repair during resolution of inflammation (Maloy *et al.* 2008; Blaschitz *et al.* 2010).

T cell phenotype plasticity

It has been observed that T cells polarised towards a Th17 phenotype can shift toward a Th1-like phenotype in response to IL-12 (Xu *et al.* 2007). Th1/Th17 cells, which release both IFN- γ and IL-17A, have also been identified (Harrington *et al.* 2005; Annunziato *et al.* 2007). Along a similar line, T cells cultured under mixed Th1 and Th2 conditions display a *continuum* of cytokine expression, with some IFN- γ^+ , some CD4 $^+$, and some double positive cells (Antebi *et al.* 2013).

Moreover, numerous data indicate that Tregs are characterised by substantial instability, especially when exposed to pro-inflammatory cytokines. It has been reported that Tregs stimulated with IL-6 can repress the expression of Foxp3 and express IL-17A, thereby acquiring the phenotype of Th17-like cells, which suggests that mature Tregs can become Th-like cells (Lee *et al.* 2009a). It has also been observed that, following peroxisome proliferator-activated receptor (PPAR) γ , Th17 cells undergo phenotype switch and become Tregs (Carbo *et al.* 2013). Furthermore, it has been observed using human naïve T cells that the addition of TGF- β to IL-1 β , IL-6 and IL-23 leads to the differentiation of Th17 cells with immunosuppressive properties (Chalmin *et al.* 2012).

Very little is known about the mechanisms underlying CD4⁺ T cell plasticity in the intestinal mucosa, however both microbial and dietary factors are likely to play an important role in this process, and the immunologic milieu surrounding a particular T cell appears to be an essential determinant for its phenotype (Brucklacher-Waldert *et al.* 2014).

Effects of TGF- β on T cell differentiation

TGF- β exerts important effects on T-LPLs. In addition to its role in Treg differentiation, TGF- β inhibits the development of intestinal Th1 cells. This is indicated by the increased frequency of colonic Th1 cells in mice with a T cell-specific deletion of *Tgfb1* gene (Li *et al.* 2007). TGF- β is essential for the prevention of Th1-mediated colitis following adoptive naïve T cell transfer in immunodeficient mice (Powrie *et al.* 1996). Culture of biopsies and lamina propria mononuclear cells (LPMCs) from human normal colon and ileum with an anti-TGF- β neutralising antibody up-regulates IFN- γ production and T-bet expression (Di Sabatino *et al.* 2008). It has been observed that TGF- β induces overexpression of micro-RNA-155 in human T-LPLs, and that this is associated with down-regulation of IL-2 and IFN- γ expression (Das *et al.* 2013).

The role of TGF- β on Th17 cell differentiation is controversial, and has been studied mainly in other tissues and organs than the intestine. In the presence of IL-6, TGF- β promotes the development of Th17 cells from murine naïve splenic T cells (Mangan *et al.* 2006; Yang *et al.* 2008) by inducing the expression of transcription factors ROR γ t and ROR α (Yang *et al.* 2008). Moreover, in the absence of TGF- β , murine Th17 cells from the spleen and lymph nodes cultured with IL-12 and IL-23 start producing IFN- γ and stop releasing IL-17A and IL-17F (Lee *et al.* 2009b). However, TGF- β blockade increases the production of IL-17A by both biopsies and LPMCs from human normal ileum and colon, which indirectly suggests an inhibitory effect of TGF- β on Th17 differentiation (Di Sabatino *et al.* 2008). It has also been hypothesised that the gene expression profile of Th17 cells may be influenced by the presence or absence of TGF- β . Indeed, stimulation of murine myelin-reactive Th17 cells with TGF- β and IL-6 abrogates their pathogenic function by inducing the production of the anti-inflammatory IL-10 despite up-regulation of IL-17A (McGeachy *et al.* 2007).

Effects of the intestinal microbiota on T-LPL differentiation

Both the presence and the composition of the intestinal microbiota exert an important influence on the number and maturation of T-LPLs. Germ-free mice have fewer T-LPLs and impaired T cell homing and, following microbial colonisation, T-LPLs return to normal levels (Round *et al.* 2009). Colonisation of germ-free mice with human intestinal microbiota restores mucosal T cell numbers only partially, and mouse intestinal microbiota is required for full immune maturation and optimal protection from *Salmonella* infection (Chung *et al.* 2012).

The symbiont *Clostridia*-related microorganism segmented filamentous bacterium (SFB) has the ability to potently induce Th17 cell differentiation in the small intestine, however SFB colonisation has protective effects on the host (Ivanov *et al.* 2009). SFB appear to colonise preferentially the surface of PPs, where they

can orchestrate mucosal T cell immunity at the inductive site. It has been shown using a bacterium-host cell co-culturing system that SFB growth *in vitro* induces a strong inflammatory and anti-microbial host response, with the transcription of TNF- α and IL-1 α and lactoferrin, and promotes an immunological environment that favours recruitment of neutrophils, T and B cells, and the transmigration of IgA (Schnupf *et al.* 2015). Colonisation of mice by a defined mix of *Clostridium* strains enhances colonic Treg number and function (Atarashi *et al.* 2011). Finally, short-chain fatty acids, such as butyrate, produced by bacteria in the large intestine, promote the development of colonic Tregs (Furusawa *et al.* 2013; Smith *et al.* 2013).

1.1.5 The humoral intestinal mucosal immune response

The humoral immune response in the intestinal mucosa is mainly characterised by the production of antigen-specific SIgA by lamina propria plasma cells, and plays an important role in maintaining mucosal immune homeostasis (Macpherson *et al.* 2005).

Differentiation of intestinal lamina propria plasma cells

Plasma cells are relatively abundant in the healthy intestinal lamina propria, and derive from B cells primed by luminal antigens in the GALT, a process which triggers B cell proliferation and differentiation, somatic hypermutation with commitment to IgA production by class switch recombination, and induction of regional homing molecules to specific parts of the intestine (Brandtzaeg *et al.* 2008). During this process, activated B cells gradually lose CD19, CD20 and B cell receptor (BCR) expression (Brandtzaeg *et al.* 2013). B cells then enter lymphatic and blood vessels and, after about 14 days since antigen exposure, enter the intestinal lamina propria and differentiate into long-lived plasma cells that release IgA (Hapfelmeier *et al.* 2010).

Interaction with the intestinal microbiota plays an important role in early B cell development in the lamina propria, in shaping the BCR repertoire, and in determining SIgA induction and composition (Hapfelmeier *et al.* 2010; Wesemann *et al.* 2013). Mucosal SIgA is nearly absent from germ-free mice (Benveniste *et al.* 1971). Upon colonisation, mice develop a long-lasting SIgA response, which is specific for the introduced species. In response to colonisation, SIgA induction occurs as a stepwise rather than a prime-boost response, and introduction of other microbial species shifts the specificity of the SIgA response against the new microbiota (Hapfelmeier *et al.* 2010).

IgA – Synthesis and release as SIgA in the intestinal lumen

Humans have two IgA subclasses (IgA₁ and IgA₂), encoded by two distinct constant region genes. While plasma IgA occurs in a monomeric, four chain form, with two heavy α chains and two light κ or λ chains, in the intestinal lumen the most abundant form of IgA is SIgA, formed by two (IgA dimers) or four (IgA polymers, pIgA) monomeric IgA, one molecule of J chain and an additional glycoprotein, the secretory component (SC), acquired during transcytosis through IECs (Kaetzel *et al.* 2013). The daily production of IgA exceeds that of all other Ig types, and more than 50% of the total IgA in the human body is synthesised in the intestinal lamina propria and actively released as SIgA in the lumen.

IgA are released in the intestinal lumen as SIgA via transcytosis through IECs. The model for the common epithelial transcytosis of IgA dimers and pIgA was proposed in 1974 (Brandtzaeg 1974). In particular, once released in the lamina propria, IgA dimers and pIgA bind to the polymeric Ig receptor (pIgR) on the basolateral surface of IECs. The pIgR-bound IgA is subsequently translocated via transcytotic vesicles to the luminal surface of IECs, where the pIgR is cleaved from the cell membrane and becomes the SC of SIgA, and ultimately the pIgR-IgA complex is released in the intestinal lumen as SIgA (Brandtzaeg 1974; Mostov *et al.* 2003). Normally, pIgR expression by IECs is high in order to maintain an adequate release of IgA in the lumen, and is regulated by both microbial and host factors. The expression of pIgR is reduced in IECs of germ-free mice, and is up-regulated by bacterial-derived butyrate and by pro-inflammatory cytokines, including IFN- γ and TNF- α , produced in response to bacterial antigens (Schneeman *et al.* 2005).

Effects of TGF- β on the induction of SIgA

TGF- β plays an important role in inducing the production of SIgA (Cerutti *et al.* 2008; Cong *et al.* 2009). This has been demonstrated in different experimental

models: mice deficient for the inhibitory protein Smad7 show enhanced isotype switch to IgA (Li *et al.* 2006), and this process, conversely, is impaired in *Smad2*^{-/-} mice (Klein *et al.* 2006). Accordingly, *TGF-β receptor (TβR)II*^{-/-} mice exhibit impaired SIgA responses both at the steady state and upon antigen stimulation, both systemically and in the intestine (Cazac *et al.* 2000). TGF-β induces IgA class switch in cooperation with B cell activating-factor of the TNF family (BAFF), a proliferation-inducing ligand (APRIL), and IL-10 (Cerutti *et al.* 2008). Moreover, TGF-β has a synergistic effect with retinoic acid, leading to enhanced IgA switch (Watanabe *et al.* 2010; Seo *et al.* 2013). DCs promote TGF-β-mediated induction of IgA class switch by up-regulating TβRII expression on B cells through the production of inducible nitric oxide synthase (Tezuka *et al.* 2007).

Functional properties of intestinal SIgA

Intestinal SIgA reduce absorption of antigens from mucosal surfaces, a process called immune exclusion (Macpherson *et al.* 2015). In particular, SIgA can prevent microorganisms from gaining access to the epithelium by a sequential process involving formation of microbial aggregates (agglutination) through binding and cross-linking, entrapment in mucus, and clearance through peristalsis (Mantis *et al.* 2010). Of note, IL-21 produced by from microbiota-specific Th17 promotes B cell trafficking to the intestine and SIgA production (Cao *et al.* 2015). On the other hand, certain pathogens can target SIgA to evade host defense. Despite the SC provides relative protection from proteolytic degradation and, unlike in peripheral blood, SIgA have a longer half-life compared to IgG in the protease-rich intestinal environment (Kaetzel *et al.* 2013), particular strains of *Neisseria gonorrhoeae*, *Haemophilus influenzae* and *Streptococcus pneumoniae* can secrete proteolytic enzymes that cleave specific post-proline peptide bonds within the extended hinge region of IgA₁ (Senior *et al.* 2000). The ability to release IgA-cleaving proteases is associated with virulence, as non-pathogenic strains are unable to produce proteolytic enzymes. Conversely, IgA₂ lacks the susceptible aminoacid sequence, hence it is resistant

to proteolysis (Senior *et al.* 2000; Bonner *et al.* 2009). Moreover, IgA-cleaving proteases can inhibit phagocytic killing of *S. pneumoniae* by IgA₁, but not IgA₂, human anti-capsule monoclonal antibodies (Janoff *et al.* 2014).

IgG transport and function in the intestinal mucosa

While SIgA transport is unidirectional, the neonatal Fc receptor (FcRn), which is expressed in IECs throughout life, is responsible for the bidirectional transport of IgG between the lamina propria and the intestinal lumen (Pyzik *et al.* 2015). FcRn is expressed mostly in endosomes, and protects internalised IgG from degradation by diverting it away from lysosomes, thereby increasing half-life of circulating IgG (Akilesh *et al.* 2007). Unlike pIgR, FcRn is not cleaved once IgG is released, hence every FcRn molecule can mediate transport of multiple IgG. FcRn-mediated IgG transport is an important mechanism of antigen delivery to the mucosal immune system (Pyzik *et al.* 2015), and on the other hand it has been shown that pathogen-specific IgG are transported into the intestinal lumen by the FcRn (Yoshida *et al.* 2006).

B cells, but not SIgA, are required for clearance of *Citrobacter rodentium*, a Gram-negative pathogenic bacterium widely used to model human infections with the attaching-and-effacing (A/E) human pathogens enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) (Maaser *et al.* 2004; Collins *et al.* 2014). The importance of mucosal IgG in the clearance of pathogens is illustrated by the observation that IgG specific for a virulence factor mediating epithelial attachment is released in the intestinal lumen of mice infected with *C. rodentium*, promoting engulfment and elimination by neutrophils. Conversely, phenotypically avirulent pathogens, which are unable to adhere to the epithelium, are eventually outcompeted by the intestinal microbiota (Kamada *et al.* 2015).

1.1.6 Oral tolerance to food and microbial antigens

Oral tolerance can be defined as a state of both local and systemic unresponsiveness to orally administered and luminal antigens, such as dietary and microbial components (Brandtzaeg 2011; Pabst *et al.* 2012). The two main mechanisms by which oral tolerance is induced are the generation of Tregs, and the clonal deletion and anergy of T cells, depending on the dose of antigen administered.

Main mechanisms of oral tolerance

In human healthy volunteers, feeding with keyhole limpet haemocyanin (KLH), an antigen used in earlier studies of oral tolerance, has been shown to suppress the antigen-specific Th1 response of subsequent parenteral immunisation (Kapp *et al.* 2010). The first event in the induction of oral tolerance is the antigen uptake from the intestinal lumen. This occurs not only in GALT, but also in the epithelium overlying effector sites, and it has been observed that antigen-loaded CD11c⁺ DCs are present in mouse lamina propria within minutes from dextran or ovalbumin feeding (Chirido *et al.* 2005). Subsequently, antigens disseminate via lymph or blood and generate systemic tolerance, and the key event for this is antigen transport by CD103⁺ DCs to the MLNs, where Treg generation occurs (Pabst *et al.* 2012). CD103⁺ DCs are particularly potent in inducing the development of Tregs and in promoting the expression of intestinal homing molecules on T cells (Sun *et al.* 2007; Jaensson *et al.* 2008). Upon generation in the MLNs, Tregs can then enter the systemic circulation via lymphatic vessels, and ultimately home to the intestinal mucosa where they exert their suppressive function on effector T cells. Oral tolerance requires both the generation of Tregs in MLNs and β_7 -dependent ability of Tregs to home to the intestinal lamina propria (Hadis *et al.* 2011). Accordingly, CCR9-deficient mice display impaired oral tolerance, which can be restored upon transfer of wild-type T cells (Cassani *et al.* 2011). Within the lamina propria, Tregs undergo secondary expansion, and

local IL-10 is needed to maintain mucosal Treg function, both in the small bowel and in the colon (Murai *et al.* 2009; Hadis *et al.* 2011).

Exposure to a high dose of antigen is considered to induce oral tolerance via another mechanism, namely clonal deletion and anergy of T cells (Weiner *et al.* 2011), however it has been observed that Tregs can also be generated when using high doses of antigen (Siewert *et al.* 2008).

Oral tolerance to the intestinal microbiota

While oral tolerance to dietary components occurs in the small intestine, immune tolerance to the intestinal microbiota is mainly induced in the colon (Pabst *et al.* 2012). Upon recognition of the intestinal microbiota, the mucosal immune system should trigger a stimulatory response in the case of pathogens, and a tolerogenic response in the case of commensal species (Chistiakov *et al.* 2015). Colonisation of germ-free mice with *Bacteroides fragilis* promotes the conversion of CD4⁺ T cells into IL-10-producing Tregs (Round *et al.* 2010). Similarly, colonisation of germ-free mice with *Clostridium*-containing human microbiota promotes the differentiation of mucosal IL-10-producing Tregs, thereby ameliorating trinitrobenzenesulfonic acid (TNBS)-induced colitis (Atarashi *et al.* 2011; Atarashi *et al.* 2013). Interaction with the colonic commensal microbiota results in the preferential generation of antigen-specific Tregs rather than effector T cells, and the TCR repertoire of colonic Tregs is influenced by the composition of the intestinal microbiota (Lathrop *et al.* 2011; Cebula *et al.* 2013). Regular stimulation by microbial antigens contributes to maintaining Tregs in the lamina propria, as suggested by the decrease in Treg proliferation in MLNs and GALT of mice treated with long-term antibiotics; however, this mechanism does not appear to be dependent on TLR stimulation, since mice deficient for various TLRs show normal, or even enhanced, Treg development (Atarashi *et al.* 2011; Cording *et al.* 2013).

Additional mechanisms contributing to oral tolerance

During homeostasis, a number of mechanisms are in place to prevent abnormal intestinal immune activation following the interaction with the components of the luminal content. Colonic IECs discriminate between different commensal bacteria by producing different cytokine patterns, and the probiotic *Lactobacillus rhamnosus* can suppress pro-inflammatory cytokine production induced by other commensals (Lan *et al.* 2005). Moreover, colonic IECs have the ability to suppress the proliferation and to prevent activation of T cells (Cruickshank *et al.* 2004). In the normal intestinal epithelium, TLR2 and TLR4 are expressed at low levels on the apical surface of IECs, thereby limiting the interaction with the intestinal microbiota and consequent possibility of an aberrant stimulatory immune response (Abreu *et al.* 2003). TLR5 is expressed only on the basolateral colonic IEC membrane, in the optimal position to detect flagellin and trigger an immune response only in case of epithelial injury (Gewirtz *et al.* 2001). Moreover, differentiated IECs have the ability to dampen the potential pro-inflammatory effects of PRR-mediated signals received from the apical side, whereas PRR signalling from the basolateral side stimulates the release of defensins in order to combat the infection (Artis 2008).

Extraintestinal effects of oral tolerance

Oral tolerance exerts an important influence on immune response not only in the intestinal mucosa, but also systemically and in other organs. Oral administration of polysaccharide A from *B. fragilis* protects mice from experimental autoimmune encephalomyelitis, with a process mediated by the induction of IL-10-producing Tregs in GALT (Ochoa-Repáraz *et al.* 2010). *B. fragilis* polysaccharide A has also been shown to enhance the development and the suppressive action of human Tregs *in vitro* (Telesford *et al.* 2015). Induction of oral tolerance to the triggering autoantigen has proved to be effective in the prevention of both experimental autoimmune encephalomyelitis, induced by immunisation with

myelin basic protein, and experimental arthritis, induced by collagen type II (Faria *et al.* 2006). However, autoimmune disease in humans is typically characterised by response to multiple autoantigens. This potential limitation to the application of oral tolerance is circumvented by the bystander suppression of Tregs, which, despite bearing an antigen-specific TCR, can inhibit local T cells in an antigen non-specific manner (Elson *et al.* 2013).

Another important limitation to the application of oral tolerance in human autoimmune disease is that the immune response to the autoantigen has already been established. It has however been shown that KLH feeding in previously immunised humans decreases the proportion of IL-17A⁺ antigen-specific T cells at the end of the feeding, and increases IL-10⁺ T cells after re-immunisation (Hostmann *et al.* 2015). The mechanisms underlying the extraintestinal effects of oral tolerance are not entirely clear, however it has been speculated that they may be mediated by a fraction of the Tregs that have expanded in the lamina propria and have then entered the systemic circulation via the draining lymphatics or via the bloodstream (Pabst *et al.* 2012).

1.1.7 Compartmentalisation of the intestinal mucosal immune system

Both the constituents and the organisation of the mucosal immune system display important distinctions between the different compartments of the gastro-intestinal tract. The largest amount of lymphoid tissue in the gastro-intestinal tract is contained in the small intestine and in the colon, which constitute structurally, immunologically and functionally distinct compartments (Bowcutt *et al.* 2014).

Structural differences between small and large intestine

The small intestine is specialised for the digestion and absorption of food, whereas the primary function of the colon is water re-absorption and elimination of undigested luminal content. The mucosa of the small intestine is organised into multiple finger-like *villi*, and the luminal side of epithelial cells is shaped into an extensive brush border of microvilli that contain digestive enzymes, hence having an optimal structural organisation for nutrient absorption. *Villi* become shorter progressing through the small intestine, and are absent in the colon. In the small intestine, crypts contain undifferentiated proliferating progenitor cells, whereas *villi* are populated by differentiated non proliferating cells. A similar organisation exists in the colon, with proliferating cells localised in the lower part of the crypt, and differentiated cells found at the epithelial surface. Compared to those present in the small intestine, colonic crypts are smaller (Mowat *et al.* 2014).

Compartmentalisation of intestinal lymphoid structures

PPs are localised on the antimesenteric side of the small intestine and increase in size and density from the jejunum to the ileum. PPs are particularly concentrated in the distal ileum, are rare in the duodenum (Cornes 1965), and are likely to be the main source of IgA-producing plasma cells that migrate to the small intestine (Masahata *et al.* 2014). Conversely, caecal and colonic patches

are important in the generation of IgA-producing plasma cells that migrate to the colon in response to the local intestinal microbiota (Masahata *et al.* 2014). The human small intestine contains at least 30,000 ILFs, increasing in density distally. In particular, in the healthy jejunum and ileum there is on average 1 ILF per 269 *villi* and 1 ILF per 28 *villi*, respectively. The density of ILFs increases distally also in the colon (Brandtzaeg *et al.* 2008). Also MLNs draining the small intestine and the colon have been shown to be anatomically and functionally separate, and the DCs that migrate to either ileal or colonic MLNs are immunologically different (Houston *et al.* 2015).

Compartmentalisation of intestinal specialised epithelial cells

Paneth cells are only present in the small intestine, and they are particularly concentrated in the ileum. In the small intestine, α -defensins secreted by Paneth cells are the major anti-microbial peptides, whereas β -defensin 2 is highly expressed in the colon during inflammation and infections (O'Neil *et al.* 1999). The frequency of goblet cells increases progressively through the gastrointestinal tract, with goblet cells being 25% of all epithelial cells in the colon, and 10% or less in the upper small intestine. In addition to the different organisation into a single layer and a double layer, also mucus composition differs substantially between the small and large intestine, however at both sites it is built on MUC2 mucin polymers (Birchenough *et al.* 2015). The mucus system is thickest in the colon, where the bacterial load is particularly abundant (Macpherson *et al.* 2013).

Compartmentalisation of intestinal mucosal T cells and plasma cells

IELs are progressively less frequent in the small intestine and relatively rare in the colon. Most IELs in the jejunum are $\alpha\beta$ TCR⁺CD8 $\alpha\beta$ ⁺ tissue-resident effector memory IELs, whereas the ileum and colon contain proportionally more $\alpha\beta$ TCR⁺CD4⁻CD8⁻ IELs (Lundqvist *et al.* 1995). In the lamina propria, there are

marked differences in the distribution and function of CD4⁺ T cells along the intestine, and this may be due, at least in part, to variations in luminal content (Sathaliyawala *et al.* 2013). An inverse correlation between lamina propria Th17 cells and Tregs has been reported in mice, with Th17 cell number progressively decreasing from the duodenum to the colon, and Treg number being highest in the colon (Denning *et al.* 2011). Conversely, in humans, a higher proportion of Th17 cells has been reported in the colon and ileum compared to the jejunum (Sathaliyawala *et al.* 2013). The frequencies of lamina propria Th1 and Th2 cells do not seem to vary significantly along the human intestine (Wolff *et al.* 2012). The density of plasma cells is highest at the most proximal and distal ends of the gastro-intestinal tract (Brandtzaeg 2010). Most of the plasma cells in the duodenum and jejunum produce IgA₁, whereas the proportion of IgA₂ increases from approximately 25% in the small intestine to >60% in the distal colon (Lin *et al.* 2014).

Compartmentalisation of PRR and adhesion molecule expression in the intestine

Also the expression of PRRs varies throughout the intestine, and these differences are likely to be driven by the intestinal microbiota (Mowat *et al.* 2014). In particular, in mice TLR2 is highly expressed on IECs of the proximal colon, whereas it is present at low levels distally (Wang *et al.* 2010). Conversely, murine TLR4 and its co-receptor CD14 are expressed at higher levels in the colon than in the small intestine (Ortega-Cava *et al.* 2003).

Several adhesion molecules and chemokines play an important role in immune cell compartmentalisation within the intestine. In particular, CCL25 is expressed by IECs in the small intestine, and its interaction with CCR9 is important in driving immune cell homing to the small intestine, but not the colon (Stenstad *et al.* 2007). Conversely, CCL28, which interacts with CCR10, is mostly expressed by colonic and rectal IECs (Pan *et al.* 2000). CCL20 and CCL9 are constitutively expressed by the FAE overlying PPs in the small intestine (Mowat *et al.* 2012).

1.2 Proteases and their inhibitors

According to their mechanisms of action and their three-dimensional structure, proteases can be classified into metalloproteinases, serine proteases, cysteine proteases and aspartic proteases (Table 1.1).

Metalloproteinases – MMPs and Adamalysins

Metalloproteinases are a group of enzymes that exert an important role in tissue remodelling through their ability to digest the ECM (Sorokin 2010). Amongst the most important enzymes in this group are MMPs and adamalysins (Huxley-Jones *et al.* 2007).

MMPs require Ca^{2+} for stability, cleave specific peptide bonds via a functional domain at neutral or near neutral pH, and contain both a catalytic domain with a highly conserved zinc-binding sequence essential for their action and a pro-domain which maintains the enzyme in inactive form and is cleaved by trypsin, plasmin, plasminogen or active MMPs during the activation process (Nagase *et al.* 2006). Currently, 24 different mammalian MMPs have been identified, and can be subdivided into MMPs that are secreted in the ECM and those that are anchored to the cell surface by a transmembrane domain (MT), such as MMP-14, also known as MT1-MMP (Parks *et al.* 2004). Another subdivision of MMPs can be made, based on their primary substrate specificity, such as collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, MMP-11), matrilysin (MMP-7) and elastase (MMP-12).

Table 1.1. Characteristics of the four major classes of mammalian proteases

Protease class	Main members	Alternative name	Main (matrix; non-matrix) substrates		
Metalloproteinases	MMPs	MMP-1	Collagenase 1	Collagen (types I-V, IX)	
		MMP-2	Gelatinase A	Gelatin, laminin, elastin, fibronectin; CCL7, CXCL12, pro-IL-1β	
		MMP-3	Stromelysin 1	Collagen (types IV, V, IX, X), laminin; IgG ₁ , pro-IL-1β, IL-1β	
		MMP-7	Matrilysin	Elastin, laminin: E-cadherin, tmTNF-α, IgG ₁	
		MMP-8	Collagenase 2	Collagen (types I-V, IX)	
		MMP-9	Gelatinase B	Gelatin, laminin; α ₁ -antiproteinase, pro-TGF-β, pro-IL-1β	
		MMP-10	Stromelysin 2	Gelatin, fibronectin	
		MMP-11	Stromelysin 3	Unknown	
		MMP-12	Macrophage metalloelastase	Elastin, collagen type IV; IgG ₁ , tmTNF-α	
		MMP-13	Collagenase 3	Collagen (types I-V, IX), gelatin	
		MMP-14	MT1-MMP	Collagen (types I-III), gelatin, fibronectin, syndecan-1	
		MMP-15	MT2-MMP	Fibrin	
		MMP-16	MT3-MMP	Fibrin, syndecan-1	
		MMP-17	MT4-MMP	Unknown	
		MMP-20	Enamelysin	Amelogenin	
		Adamalysins	ADAM8		Unknown; L-selectin
			ADAM9		Unknown; pro-EGF
			ADAM10	CD156c	Unknown; E-cadherin, tmTNF-α, pro-TGF-α, DSGs, HER2
			ADAM15		Unknown; E-cadherin
			ADAM17	TNF-α converting enzyme	Unknown; tmTNF-α, pro-TGF-α, pro-EGF, DSGs, IL-6R, L-selectin
ADAM19			Unknown; tmTNF-α		
Serine proteases	Plasminogen activators		Aggrecan, syndecan, fibronectin, laminin; plasminogen, trypsinogen, HGF, pro-TGF-β		
	Trypsin		Unknown; trypsinogen, lysine-, arginine-containing proteins, α-defensin		
	Chymotrypsin		Unknown; tryptophan-, tyrosine-, phenylalanine-, leucine-containing proteins		
	Cathepsin G		Unknown; lysine-, arginine-, tryptophan-, tyrosine-, phenylalanine-, leucine-containing proteins		
	Neutrophil elastase	ELA2	Elastin		
Cysteine proteases	Cathepsin B		β-amyloid precursor protein, proteoglycans, collagen, connective tissue proteins		
	Cathepsin L		Collagen type I		
	Cathepsin N		β-amyloid precursor protein		
	Cathepsin S		Elastin		
	Calpains		Unknown; STIM1		
	Caspases		Unknown; Bax, Bcl-2, pro-IL-1β, procaspases 1-14		
Aspartic proteases	Pepsin		β-amyloid invariant chain, IgG		
	Cathepsin D		MHC class II		
	Renin		Unknown; angiotensinogen		

HER, human epidermal growth factor receptor; HGF, hepatocyte growth factor; IL-6R, IL-6 receptor; MT, transmembrane domain; tm, membrane-bound.

Most MMPs are released as inactive zymogens by epithelial cells and mesenchymal cells, monocytes, macrophages, neutrophils and T cells. MMP activation occurs in the extracellular space and is mediated by the cleavage of the pro-domain that maintains the molecule in its inactive form. This process is amplified by a cascade of autocatalytic reactions and cleavage processes by which MMPs can activate each other (Fig. 1.1) (Knäuper *et al.* 1993; Fridman *et al.* 1995; Knäuper *et al.* 1996; Makowski *et al.* 2005).

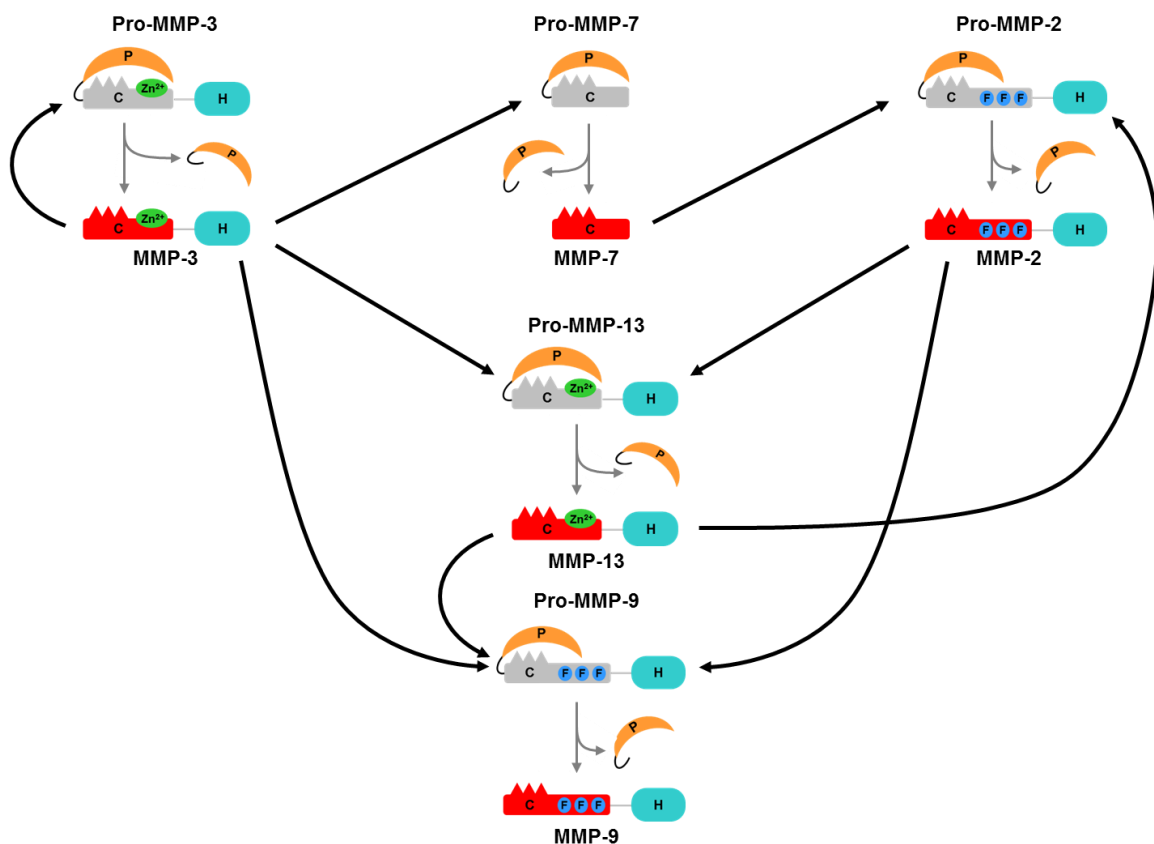


Figure 1.1

Figure 1.1. Representative example of MMP activation process. Pro-MMPs are converted to the active form via proteolytic removal of their pro-domain. Active MMP-3 can amplify its own activation via autocatalytic cleavage, or alternatively can remove the pro-domain from pro-MMP-7, pro-MMP-9 and MMP-13. Active MMP-7, in turn, exerts an activating action on pro-MMP-2, and active MMP-13 can activate both MMP-2 and MMP-9. Moreover, active MMP-2 can cleave MMP-13 and MMP-9 pro-domain. As shown in this example, proteolytic enzymes can therefore auto-activate themselves, mutually activate each other and trigger a cascade of protease activation processes. All these mechanisms

can lead to a broad proteolytic activation in diseased tissues. C, catalytic domain; F, fibronectin repeat; H, haemopexin-like domain; P, pro-domain. (From Biancheri *et al.* 2013).

MMP proteolytic activity is regulated by differential transcription, activation, substrate availability and inhibition (Di Girolamo *et al.* 2006; Sorokin 2010). The latter is mediated by the four tissue inhibitors of metalloproteinases (TIMP)1-4, whereas the major endogenous serum MMP inhibitor is α 2-macroglobulin (Sorokin 2010). ECM degradation and regulation of tissue turnover are not the only important functions of MMPs, which also exert their proteolytic action on non-structural molecules embedded within the matrix, leading to their activation or facilitating their catabolism (Parks *et al.* 2004). In particular, MMPs can act on intercellular junction proteins, chemokines, cytokines, IgG₁ and microorganisms, potentiating or inhibiting their activity (Table 1.2). The effect of MMPs on cytokines is diverse. For example, MMP-2, MMP-3 and MMP-9 can cleave and activate the IL-1 β precursor, however MMP-3 can also degrade the active form of this cytokine (Ito *et al.* 1997; Schönbeck *et al.* 1998).

Adamalysins are a family of transmembrane proteinases crucially involved in ectodomain shedding of growth factors, cytokines, membrane receptors and adhesion molecules (Edwards *et al.* 2008). One of the most studied adamalysins is a disintegrin and a metalloproteinase (ADAM)17, also known as TNF- α converting enzyme (TACE), a membrane-bound enzyme which cleaves the transmembrane form of TNF- α to generate soluble TNF- α , and which is also essential for the shedding of other cell surface proteins such as TGF- α , IL-6 receptor and L-selectin (Scheller *et al.* 2011), thus playing an important role in the control of inflammatory and regenerative responses.

Table 1.2. Main substrates of MMPs

Type of substrate	Molecule/species	MMPs	Effect
ECM components	Collagen I-IV	MMP-1, MMP-3, MMP-7, MMP-8, MMP-10, MMP-12, MMP-13, MT1-MMP	Degradation
	Collagen V	MMP-1, MMP-3, MMP-8, MMP-13	Degradation
	Collagen IX	MMP-1, MMP-3, MMP-8, MMP-13	Degradation
	Collagen X	MMP-3	Degradation
	Elastin	MMP-2, MMP-7, MMP-12	Degradation
	Laminin	MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, MMP-12	Degradation
	Fibronectin	MMP-2, MMP-3, MMP-7, MMP-10, MMP-12, MT-MMP-1	Degradation
	Gelatin	MMP-2, MMP-9, MMP-10, MMP-13, MT1-MMP	Degradation
	Proteoglycan	MMP-3, MMP-7, MMP-10	Degradation
	Chondroitin sulfate	MMP-12	Degradation
	Aggrecan	MMP-2, MT1-MMP	Degradation
Intercellular junction proteins	E-cadherin	MMP-7, enterotoxin, gelatinase E	Degradation
Cytokines/chemokines	Pro-IL-1 β	MMP-2, MMP-3, MMP-9	Activation (by cleavage)
	IL-1 β	MMP-3	Inactivation (by cleavage)
	tmTNF- α	MMP-7, MMP-12, MMP-13	Conversion into soluble TNF- α
	CXCL5/CXCL6	MMP-8	Activation (by cleavage)
	CXCL8 (IL-8)	MMP-9	Activation (by cleavage)
	CCL7	MMP-2	Inactivation (by cleavage)
	CXCL1	MMP-7	Generation of a chemotactic gradient by cleavage of CXCL1 ligand syndecan-1
Antibodies	IgG ₁	MMP-3, MMP-7, MMP-12	Cleavage
Bacteria	<i>Staphylococcus aureus</i>	MMP-12	Inhibition
	<i>E. coli</i>	MMP-12	Inhibition
	<i>C. rodentium</i>	MMP-3	Increased clearance via regulation of T cell migration

tm, membrane-bound.

Serine proteases

Another class of proteases is represented by serine proteases, which exert their action at neutral pH. Mammalian serine proteases include plasminogen activators, chymotrypsin, trypsin, and proteolytic enzymes produced by granulocytes and mast cells, such as cathepsin G and neutrophil elastase. Bacteria also produce serine proteases, such as subtilisin (Hedstrom 2002).

Cathepsin G and neutrophil elastase are stored in granules as inactive precursors and released as active forms in response to inflammatory or allergic reactions. Serine protease inhibitors, also known as serpins, include α 1-antitrypsin, α 1-antichymotrypsin, antithrombin III, elafin, protease-nexin and the universal protease inhibitor α 2-macroglobulin (Silverman *et al.* 2001).

Cysteine proteases

Cysteine proteases include mammalian lysosomal cathepsins B, K, L, N and S, which can degrade connective tissue proteins *in vitro*, and cytosolic calcium-activated proteases, also known as calpains (Zavasnik-Bergant *et al.* 2006). These proteases are inhibited by cystatins, a superfamily including stefins, cystatin C and S and kininogens (Abrahamson *et al.* 2003). Caspases are intracellular cysteine-aspartic proteases involved in apoptosis, necrosis, and inflammation. Caspases, such as caspase-3, cleave a wide range of intracellular proteins, thereby inducing apoptotic cell death. Caspases are specifically associated with receptor-activated apoptosis, for example Fas/FasL-mediated programmed cell death. Finally, caspases, such as caspase-9, initiate a cascade of cleavage of other caspases, thereby activating them (Becker *et al.* 2013).

Aspartic proteases

Finally, aspartic proteases include bacterial penicillopepsin and mammalian pepsin, cathepsin D, and renin (Tang *et al.* 1997). Cathepsin D is involved in the enzymatic removal of invariant chain from class II MHC molecule during antigen processing and in the production of antigenic peptides in the phagolysosome of APCs (Villadangos *et al.* 1999). Both cysteine proteases and aspartic proteases exert their action at low pH values and are therefore often referred to as acidic proteases.

1.2.1 Proteases in intestinal homoeostasis and wound healing

Proteases are constitutively expressed in the healthy human intestine, where they are produced mainly by IECs and by goblet cells in the epithelium, by immune and mesenchymal cells in the lamina propria, and by bacteria in the lumen (Ravi *et al.* 2007; Steck *et al.* 2012). Proteases are essential to preserve mucosal homoeostasis and physiologic tissue turnover, and are central players in the process of wound healing and re-epithelisation after injury. Moreover, proteases may exert important effects on the epithelial barrier (Fig. 1.2).

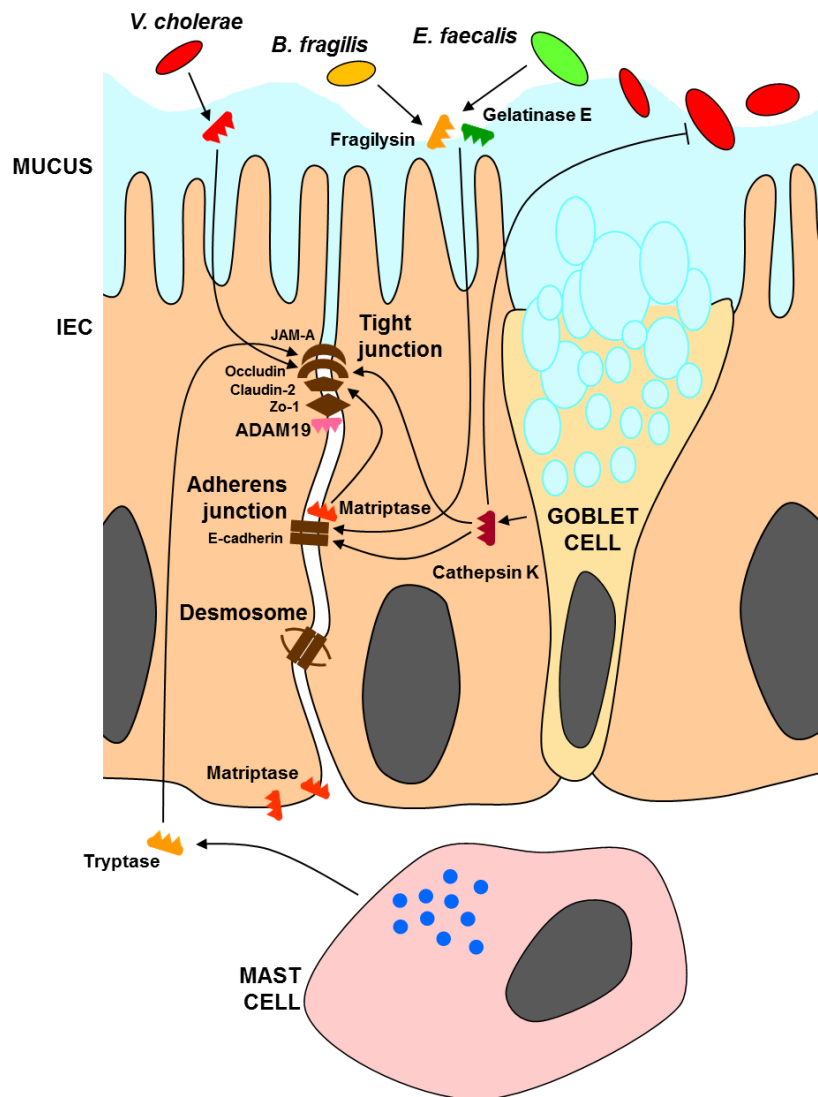


Figure 1.2

Figure 1.2 (see previous page). **Effects of intestinal proteases on proteins forming intercellular junctions in the intestinal epithelium.** Proteases can exert their actions on several elements of the intercellular junctions between IECs. In particular, matriptase, a trypsin-like transmembrane serine protease, co-localises with E-cadherin in the adherens junction and is also present on the basolateral side of IECs. Matriptase regulates the expression of epithelial claudin 2, a junctional protein which increases epithelial permeability. Another transmembrane protease, ADAM19, co-localises with zonula occludens-1 (Zo-1), a tight junction-associated protein. Goblet cells release the cysteine protease cathepsin K, which regulates E-cadherin and occludin expression, and protects from colitis by means of its potent anti-microbial activity. Activated mast cells in the lamina propria release the proteolytic enzyme tryptase, which decreases epithelial barrier function by reducing the expression of the tight junction protein junctional adhesion molecule (JAM)-A. Pathogenic bacteria (red), as well as opportunistic pathogens (yellow) and commensal bacteria (green) can release proteases which have important effects on components of the intercellular junctions. *Vibrio cholerae* produces haemagglutinin/protease, which can degrade occluding. Moreover, both the metalloproteinase gelatinase E, produced by the commensal bacterium *Enterococcus faecalis*, and the MMP fragilylin, released by *B. fragilis*, are capable of cleaving E-cadherin in the adherens junction. (Adapted from Giuffrida *et al.* 2014).

Proteases produced by IECs

Constitutive epithelial expression of matrilysin (MMP-7) has been shown to mediate defense against luminal bacteria in mice. In particular, mouse MMP-7 activates intestinal pro- α -defensins, needed for the clearance of pathogenic *E. coli* and *S. typhimurium* (Wilson *et al.* 1999). MMP-7 can cleave transmembrane FasL, which induces apoptosis of target cells by binding to Fas receptor expressed on IEC surface (Powell *et al.* 1999). Epithelial MMP-7 expression is dependent on the exposure to intestinal bacteria (López-Boado *et al.* 2000; Salmela *et al.* 2004), essentially priming the epithelium for anti-bacterial activity. Moreover, IECs constitutively express high levels of meprin, a metalloproteinase which counteracts bacterial adhesion and invasion, thus representing an important host defense mechanism against pathogenic strains of *E. coli* (Vazeille *et al.* 2011).

After injury, collagenase 1 (MMP-1), MMP-7 and stromelysin 2 (MMP-10) are expressed by migrating IECs and promote re-epithelisation by facilitating the migration of IECs toward the wound edge (Salmela *et al.* 2004). *MMP-7^{-/-}* mice show a complete lack of re-epithelisation after wounding (Parks *et al.* 2004). During lung injury, MMP-7 has been shown to degrade E-cadherin from adherens junctions, consequently promoting cell migration and tissue remodeling, however these findings have not been replicated in the intestine (McGuire *et al.* 2003). Epithelium-derived MMP-7 can generate a chemotactic gradient by cleaving from the epithelial cell surface the proteoglycan syndecan-1, the ligand for the neutrophil chemoattractant CXCL1 (Li *et al.* 2002). MMP-1, MMP-7 and MMP-10 expression by IEC cell lines is up-regulated by EGF, TGF- β and IL-1 β (Salmela *et al.* 2004).

Matriptase is an epithelial trypsin-like transmembrane serine protease that co-localises with E-cadherin in the apical junctional complex, is also present on the basolateral aspect of the cell, and is activated by a glycosylphosphatidylinositol-anchored serine protease, prostasin (Buzza *et al.* 2010; Buzza *et al.* 2013). In mice, matriptase is encoded by *St14* gene, and it has been shown that IEC-specific *St14^{-/-}* mice develop diarrhoea from birth, show megacolon and colitis and die early in life, however the small intestine is normal (List *et al.* 2009). Tamoxifen-inducible IEC-specific *St14^{-/-}* mice become moribund 9-10 days after tamoxifen treatment. These animals show severe oedema and dissolution of the colonic mucosa and regenerative crypt hyperplasia, but the small intestine is normal (List *et al.* 2009). Mice hypomorphic for the *St14* gene are viable but show increased permeability in the small intestinal barrier (Buzza *et al.* 2010). The addition of exogenous matriptase to the basolateral side of Caco-2 cells markedly increases transepithelial resistance, whereas when *matriptase* is knocked down in Caco-2 cells there is a decrease in transepithelial resistance and an increase in paracellular permeability (Buzza *et al.* 2010; Buzza *et al.* 2013). Interestingly, knock-down of *matriptase* results in an increase in epithelial claudin 2, a tight junction protein known to enhance epithelial permeability

(Prasad *et al.* 2005; Buzza *et al.* 2010). Matriptase-hypomorphic mice are also particularly susceptible to DSS-induced colitis (Netzel-Arnett *et al.* 2012).

Proteases produced by intestinal specialised epithelial cells

Intestinal goblet cells produce the acidic cysteine protease cathepsin K, which has activity also at neutral pH and can degrade elastin, collagen, and gelatin. *Cathepsin K*^{-/-} mice have increased amounts of type IV collagen in the intestinal epithelial basement membrane, increased E-cadherin at the apical junctions and disrupted expression of occludin (Arampatzidou *et al.* 2012). *Cathepsin K*^{-/-} mice are significantly more susceptible to DSS colitis, possibly because cathepsin K has potent anti-microbial activity, and intrarectal administration of cathepsin K ameliorates DSS colitis (Sina *et al.* 2013).

In the normal small bowel, Paneth cells activate caspase-8 to prevent IEC necroptosis (Becker *et al.* 2013). Mice with a conditional deletion of caspase-8 in intestinal cells of epithelial lineage show increased TNF- α -induced necroptosis and death of IECs and Paneth cells, spontaneously develop an inflammatory response in the terminal ileum and are highly susceptible to experimental colitis (Günther *et al.* 2011). Loss of Paneth cells, in turn, impairs anti-microbial defense and intestinal barrier function, potentially leading to the abnormal ingress of bacteria and, subsequently, immune activation (Becker *et al.* 2013).

Proteases produced in the intestinal lamina propria

During homeostasis, mesenchymal cells and immune cells in the lamina propria release small, detectable amounts of stromelysin 1 (MMP-3) (Ravi *et al.* 2007). MMP-3 seems to be involved in T cell migration, as shown by the observation that *MMP-3*^{-/-} mice infected with *C. rodentium* show an impaired migration of CD4⁺ cells in the lamina propria and a delayed bacterial clearance (Li *et al.* 2004). Macrophage metalloelastase (MMP-12) exerts a direct bactericidal effect on

Staphylococcus aureus and *E. coli* by disrupting bacterial cell wall, and *MMP-12*^{-/-} mice have impaired survival to intraperitoneal *E. coli* administration (Houghton *et al.* 2009).

Mast cell secretory granules contain large amounts of the cell-specific serine proteases chymases, tryptases and carboxypeptidase A (Douaiher *et al.* 2014). Mice lacking mast cells or mast cell chymase *Mcpt4* have significantly reduced basal small intestinal permeability compared to wild-type mice (Groschwitz *et al.* 2009). Upon performing kinetic analysis of IEC renewal in mast cell-deficient mice and *Mcpt4*-null mice, the reduced intestinal permeability has been found to be associated to defective IEC migration along the villus/crypt axis, altered intestinal morphology, and dysregulated claudin 3 crypt expression (Groschwitz *et al.* 2009). Studies on murine chymases are of limited applicability in humans, as there are 10 chymases in mice and only one chymase in humans (Douaiher *et al.* 2014). The addition of human chymase to Caco-2 cell monolayers *in vitro* down-regulates transepithelial resistance (Groschwitz *et al.* 2013). The increase in epithelial permeability induced by human mast cell chymase does not result from a direct effect on adherens junctions. Instead, chymase activates protease-activated receptor-2 and induces the expression of gelatinase A (MMP-2), which mediates tight junction protein claudin 2 degradation (Groschwitz *et al.* 2013). Overall, however, MMP-2 appears to have a protective effect on the epithelial barrier, since *MMP-2*^{-/-} mice show a higher susceptibility to experimental colitis and an increase in intestinal permeability (Garg *et al.* 2006). Tryptase is another mast cell-derived protease. When added to Caco-2 cell monolayers, tryptase decreases transepithelial resistance (Wilcz-Villega *et al.* 2013). This appears to be due to a reduction in the expression of tight junction protein junctional adhesion molecule-A (JAM-A) (Wilcz-Villega *et al.* 2013).

After injury, MMPs promote leukocyte recruitment to the site of lesion, an important process in order to clear infection and damaged tissue. In particular, collagenase 2 (MMP-8) and gelatinase B (MMP-9) activate by cleavage the

chemokines CXCL5/CXCL6 and CXCL8 (IL-8) respectively, thereby promoting leukocyte recruitment at the site of damage (Van Den Steen *et al.* 2003). It has also been shown that the combined action of MMP-8, MMP-9 and prolyl-endopeptidase generates collagen cleavage products, such as the tripeptide proline-glycine-proline (PGP) and its acetylated form N-acetyl-PGP, with chemoattractive effects on neutrophils (Koelink *et al.* 2014). MMPs produced in the lamina propria are involved also in the subsequent phases of tissue repair and wound healing. Upon activation, myofibroblasts express high amounts of MT1-MMP (MMP-14) and MMP-2, which increase their migration through synthetic matrix and therefore may enhance their wound healing potential (Pender *et al.* 2000). Moreover, it has been shown that MMP-14 promotes airway epithelial cell proliferation after injury (Atkinson *et al.* 2007). MMP-10, which is normally produced by stromal cells in the intestinal lamina propria, appears to be involved in wound healing and co-localises with MMP-1 at the migrating epithelial front (Ravi *et al.* 2007).

Proteases produced by the intestinal microbiota

The intestinal microbiota is an important source of proteases. In particular, both commensal and pathogenic bacteria can produce a wide range of proteolytic enzymes such as serine and aspartic proteases and metalloproteinases, which can exert profound effects on the integrity and the functions of the intestinal barrier (Steck *et al.* 2012).

It has been shown that the commensal bacterium *Enterococcus faecalis* produces the metalloproteinase gelatinase E, which can degrade the junctional proteins occludin and E-cadherin (Steck *et al.* 2011). IL-10^{-/-} mice mono-associated with gelatinase E-producing *E. faecalis* develop colitis, whereas colonic inflammation is significantly reduced in the absence of bacteria-derived gelatinase E (Steck *et al.* 2011). Disruption of the intestinal epithelial barrier by

gelatinase E-producing *E. faecalis* occurs only in genetically susceptible hosts, before the onset of intestinal inflammation (Steck *et al.* 2011).

Proteases can also be produced by opportunistic pathogens. *Clostridium perfringens* can produce collagenase A, which degrades mucus and collagen types I and IV (Pruteanu *et al.* 2011). The ability of *B. fragilis* to induce inflammatory diarrhoea depends on the production of the MMP fragilysin, also known as *B. fragilis* toxin, which can cleave E-cadherin from the surface of HT-29 cells, and can mediate the pathogenic effects of *B. fragilis* by binding to a specific receptor expressed on colonic IECs (Wu *et al.* 2007; Sears *et al.* 2014).

Both non-invasive and invasive pathogens release a wide range of proteases, which can exert important effects on the intestinal barrier and may be relevant for evading host defense mechanisms. *Vibrio cholerae* haemagglutinin/protease can degrade mucin and occludin, and thereby disrupt the intestinal barrier (Wu *et al.* 2000). The protease domain in lymphostatin released by *C. rodentium* disrupts the integrity of epithelial barrier in the intestine of infected mice (Babbin *et al.* 2009). Moreover, *C. rodentium* outer membrane protease (CroP) cleaves the murine cathelicidin-related antimicrobial peptide (CRAMP) (Brannon *et al.* 2015). The A/E human pathogens EPEC and EHEC, major causes of diarrhoeal disease worldwide, can inject into IECs the effector protein NleC, which degrades the p65 subunit of NF- κ B (Shames *et al.* 2011). EHEC produces also the metalloproteinase StcE, which can cleave MUC7 mucin and potentially facilitate adherence to IECs (Grys *et al.* 2005). It has been shown that the OmpT outer membrane protease of EHEC and, to a lesser extent, EPEC degrades the human anti-microbial cathelicidin LL-37 (Thomassin *et al.* 2012). Pic, a secreted protease released by enteroaggregative *E. coli* (EAEC) and *Shigella flexnerii*, has the ability to degrade gelatin (Henderson *et al.* 1999). Adherent-invasive *E. coli* (AIEC) produces a protease called Vat-AIEC, which facilitates crossing of the intestinal mucus layer (Gibold *et al.* 2015).

Also parasites may release proteases affecting the intestinal barrier. *Entamoeba histolytica* also secretes a cysteine protease, which dissolves the intestinal mucus layer (Lidell *et al.* 2006). The nematode *Trichuris muris* secretes a number of proteases as excretory/secretory products. Excretory/secretory products can degrade the N-terminus of MUC2 mucin, thus reducing mucus viscosity, and since this property is inhibited by chymostatin but not inhibitors of other classes of proteases it is assumed that the proteolytic activity is due to a serine protease (Hasnain *et al.* 2012). Interestingly, serpins, natural inhibitors of serine proteases, are increased in the mucus layer during worm expulsion, suggesting a role in protective immunity (Hasnain *et al.* 2012).

1.3 IBD – Clinical aspects

1.3.1 Definition and history

IBD, including Crohn's disease (CD) and ulcerative colitis (UC), is a group of chronic, inflammatory, spontaneously relapsing disorders of the gastrointestinal tract (Baumgart *et al.* 2012; Ordás *et al.* 2012). CD had been first observed by the German surgeon Wilhelm Fabry (aka Guilihelmus Fabricius Hildanus) in 1623 (Anonymous 1964). In 1761 Giovanni Battista Morgagni, in his "*De Sedibus et Causis Morborum*", gave the clinical description of a probable case of CD (Kirsner 1988). CD has been subsequently described in 1913 in six cases in which tuberculosis was excluded by careful bacteriological studies, and defined as "chronic interstitial enteritis" by the Scottish surgeon Thomas Kennedy Dalziel (Dalziel 1913), and has been later named after the US physician Burril B Crohn (Crohn *et al.* 1984). UC was first described in 1859 by the London physician Sir Samuel Wilks, who reported the case of a 42 year old woman who died after several months of diarrhoea and fever (Wilks 1859). Autopsy demonstrated ulcerative inflammation of the colon and terminal ileum which was designated as "simple UC". Later on, in 1875, Wilks and Moxon described ulcerative inflammation in the entire colon in a young lady who died after severe bloody diarrhoea (Wilks *et al.* 1875).

CD and UC have traditionally been considered a disease of developed Western countries, with the highest incidence in North America, Western Europe and Australia (Cosnes *et al.* 2011). However, since 1990 IBD have emerged in newly industrialised countries, and their prevalence is now rising in every continent (Kaplan 2015). Interestingly, the rise in UC incidence in developing countries often precedes that of CD by approximately one decade (Ananthakrishnan 2015).

Both CD and UC have a fluctuating clinical course, with periods of remission alternating to flares of disease, and are characterised by a wide clinical

heterogeneity (Cosnes *et al.* 2011; Baumgart *et al.* 2012; Ordás *et al.* 2012). Extraintestinal manifestations are present in up to 40% of patients with IBD, involve mainly the joints, the eyes and the skin, and include peripheral seronegative arthritis, sacroileitis, ankylosing spondylitis, erythema nodosum, *Pyoderma gangrenosum*, episcleritis and uveitis (Williams *et al.* 2008; Ott *et al.* 2013). By definition, CD can potentially affect any part of the gastrointestinal tract, from the mouth to the anus, however in three quarters of CD patients the lesions are localised in the terminal ileum and/or in the ascending colon. Inflammation in CD is usually patchy and transmural, as lesions can involve all the layers of the intestinal wall. Conversely, UC is a mucosal disease which always affects the rectum and can spread proximally up to the caecum with a continuous retrograde distribution, without patchiness or skip lesions (Baumgart *et al.* 2007b). The main clinical and pathological characteristics distinguishing CD and UC are summarised in Table 1.3. In approximately 5% of patients with colitis it is not possible to formulate a conclusive diagnosis of CD or UC, and this condition is termed IBD, type unclassified (IBDU) (Mowat *et al.* 2011).

Table 1.3. Clinical and pathological features of CD and UC

	CD	UC
Clinical presentation	Severe abdominal pain, mainly in right <i>iliac fossa</i> Diarrhoea Possible abdominal mass in the right <i>iliac fossa</i> (inflamed or strictured ileum) Possible obstructive symptoms (intestinal stricture)	Cramping abdominal pain, mainly in left <i>iliac fossa</i> Bloody diarrhoea Abdominal mass in the left <i>iliac fossa</i> (inflamed sigma) – Rare Rectal tenesmus, urgency
Complications	Abscesses Intestinal strictures Fistulae	Colorectal cancer Primary sclerosing cholangitis
Endoscopic features	Discontinuous, “patchy” distribution of lesions Ileal involvement Linear and serpiginous deep ulcers “Cobblestone” appearance	Continuous distribution of lesions Rectal involvement Superficial and diffuse ulcers Mucosal oedema
Histologic features	Epithelioid granulomas Mucin preservation at active sites Transmural involvement	Crypt architectural distortion Mucin depletion Basal cell plasmacytosis Mucosal involvement

1.3.2 CD

1.3.2.1 Epidemiology

The annual incidence of adult CD is up to 20.2 per 100,000 inhabitants in North America and up to 12.7 per 100,000 inhabitants in Europe, depending on different geographical areas (Ananthakrishnan 2015). In the UK, CD affects at least 115,000 patients, with a prevalence of approximately 200 in 100,000 inhabitants (Mayberry *et al.* 2013). CD has its main peak of incidence between the second and third decade, and it is 20-30% more frequent in women than in men (Cosnes *et al.* 2011; www.crohnsandcolitis.org.uk). The incidence of adult CD in the UK has been rising markedly between the 1950s and the 1980s, whereas in the subsequent decades the rate of increase has been slower (Mowat *et al.* 2011). Conversely, from 1988 to 2007 the incidence of CD in Northern France has increased by 29% in the overall population and by 71% in the 10-19 year-old age group (Chouraki *et al.* 2011), suggesting that the recent increase in incidence is primarily attributable to paediatric cases. Paediatric CD is more frequent in males than in females (Cosnes *et al.* 2011).

1.3.2.2 Diagnosis

The diagnosis of CD is based on a thorough clinical evaluation and a combination of endoscopic, histologic, laboratory and radiologic test results (Mowat *et al.* 2011).

Clinical manifestations and laboratory investigations

Clinical manifestations of CD are heterogeneous; however, they commonly include diarrhoea for more than six weeks, abdominal pain and/or weight loss (Van Assche *et al.* 2010a). In a subject with suspect CD, initial laboratory tests should include full blood count, urea and electrolytes, liver function tests,

erythrocyte sedimentation rate (ESR) and/or C reactive protein (CRP), ferritin, vitamin B12 and folate (Mowat *et al.* 2011). Faecal calprotectin, an anti-bacterial protein produced by neutrophils and activated macrophages, is a reliable marker of active colonic inflammation both in CD and UC (Mosli *et al.* 2015), and can be a useful marker in distinguishing IBD from functional diarrhoea (Mowat *et al.* 2011). Anti-*Saccharomyces cerevisiae* serum antibodies (ASCA) are present in a significant proportion of CD patients and are very rare in UC, however there is no evidence base to recommend their routine use in clinical practice (Mowat *et al.* 2011).

Endoscopic, histologic and radiologic findings

Pancolonoscopy with retrograde ileoscopy and multiple biopsies is the gold standard confirmatory exam for the diagnosis of ileo-colonic CD (Van Assche *et al.* 2010a). Typical endoscopic lesions in active CD are areas with aphtous ulcerations alternated with unaffected areas. This patchy discontinuity of lesions leads, in the most severe cases, to the development of a “cobblestone” pattern. Discontinuous distribution of the lesions, ileal involvement, and deep ulcerations are important endoscopic features in the differential diagnosis with UC. According to the guidelines of the British Society of Gastroenterology (Mowat *et al.* 2011), perendoscopic biopsy sampling (two biopsies from at least five intestinal segments including the ileum and the rectum) and histologic analysis should be performed and the differential diagnosis with UC should be attempted.

The presence of non-caseating granulomas, focal or patchy lamina proprial chronic inflammation, conserved crypt architecture and a normal number of goblet cells (mucin depletion and crypt distortion are typical in UC) help confirm the diagnosis of CD (Baumgart *et al.* 2012; Feakins 2013).

Radiological investigations are particularly useful to study the extent and complications in CD (Van Assche *et al.* 2010a). Magnetic resonance imaging

(MRI) allows rapid and accurate assessment of small bowel extent and activity of disease in CD, and may be useful in differentiating between inflammatory and fibrotic intestinal strictures (Mowat *et al.* 2011). Computed tomography (CT) enteroclysis and CT enterography provide similar information to MRI, are the gold standard for the study of abscesses, and are usually available out of hours; however, CT has the major disadvantage of high radiation exposure (Mowat *et al.* 2011).

Complications

Especially in case of clinical manifestations such as fever, tenesmus, perianal pain and subocclusive symptoms, particular attention should be dedicated to the investigation of possible complications of CD. These include intra-abdominal or intraparietal perianal abscesses, fistulae – which abnormally connect the intestinal lumen with another part of the gastro-intestinal tract, or with a contiguous organ (e.g. bladder, ureter, vagina), or with the skin – and intestinal strictures, which are caused by intestinal inflammation and/or fibrosis and lead to the subsequent development of pre-stenotic dilatations (Van Assche *et al.* 2010a).

1.3.2.3 Classification

According to the Montreal classification (Silverberg *et al.* 2005; Satsangi *et al.* 2006), CD can be classified in clinical phenotypes depending on the age of onset (Montreal A-category), the localisation of intestinal lesions (Montreal L-category), and the disease behaviour (Montreal B-category). In particular, A1 indicates diagnosis < 16 years of age, A2 17-40 years and A3 > 40 years. L1 indicates lesions localised only in the ileum, L2 only in the colon, L3 ileo-colonic disease, L4 disease in the upper gastrointestinal tract, L4+L3 upper gastrointestinal tract + distal disease. Finally, B1 indicates non-stricturing non penetrating (also known as luminal) disease, B2 stricturing (also known as fibrostenosing) disease, B3

penetrating (also known as fistulising) disease, B3p perianally penetrating disease. The Montreal classification is useful for patient stratification, as different clinical phenotypes have different treatment options (Van Assche *et al.* 2010b). While disease localisation is usually stable, the behaviour tends to change over time. In particular, the large majority of patients have luminal disease at diagnosis, whereas after 25 years of follow-up most of CD patients evolve towards a B2 or B3 disease phenotype (Cosnes *et al.* 2011).

1.3.2.4 Disease activity indices

Several clinical, laboratory, endoscopic and radiologic indices of disease activity in CD have been developed as a means to objectively evaluate, particularly in the research and clinical trial settings, the response to treatment, and with a view to ultimately stratify patients according to their predicted disease course. However, none of the disease activity indices in CD has been formally validated according to the guidance of the US Food and Drug Administration for the development of patient-reported or clinician-reported outcomes (Levesque *et al.* 2015).

Crohn's Disease Activity Index (CDAI) and Harvey-Bradshaw Index (HBI)

The CDAI has been widely used as outcome measure in clinical trials since the publication of the National Crohn's Disease Study trial in 1979 (Best *et al.* 1976). CDAI ranges from 0 to 600 according to the presence and severity of eight independent predictors (seven clinical manifestations and haematocrit). CDAI < 150 indicates disease remission; between 150 and 219, mildly active disease; between 220 and 449, moderately active disease; > 450, severely active disease (Best *et al.* 1976). CDAI correlates poorly with endoscopic inflammation, serum CRP and faecal calprotectin, as symptoms of active CD overlap with conditions such as irritable bowel syndrome, small bowel bacterial overgrowth or bile salt malabsorption (Jones *et al.* 2008).

The HBI can be described as a simplified version of CDAI, and consists of five clinical predictors (which is a useful feature when laboratory test results are not available). Remission is defined as a score < 5 (Harvey *et al.* 1980). A 3-point change in HBI corresponds with a 100-point change in CDAI, and an HBI ≤ 4 corresponds with a CDAI < 150 (Vermeire *et al.* 2010).

Endoscopic disease activity indices

In 1989 the Crohn's Disease Endoscopic Index of Severity (CDEIS) was developed based on colonoscopy findings in 75 CD patients, and showed a remarkable interobserver agreement (Mary *et al.* 1989). The CDEIS considers five intestinal segments (ileum, right colon, transverse colon, left colon/sigmoid, rectum). For each of them four parameters are evaluated and graded from 0 to 3 based on severity, namely the presence and size of ulcerations, the percentage of ulcerated surface, the percentage of inflamed surface, and the presence of strictures. The cut-off points for CDEIS have been different among different investigators. Complete endoscopic remission was subsequently defined by the same group as a score of < 3 , endoscopic remission as a score of < 6 , and endoscopic response as a decrease of > 5 points, whereas other studies have used different cut-off points (Rutgeerts *et al.* 2012; Hébuterne *et al.* 2013). In particular, mild, moderate and severe CD have been described as CDEIS < 5 , 5 to 15, and > 15 , respectively (Geboes *et al.* 2005).

In 2004, the Simple Endoscopic Score in Crohn's Disease (SES-CD) was developed in order to make objective endoscopic assessment and grading of disease severity in CD easier and more applicable to clinical practice (Daperno *et al.* 2004). The five intestinal segments considered in the SES-CD are the same as in CDEIS, however the descriptors (presence/absence of deep ulcerations, presence/absence of superficial ulcerations, surface involved by disease and

surface involved by ulcerations, with an additional score for the presence of a stricture) are more immediate to determine than those of the CDEIS.

Endoscopic mucosal healing in CD has been defined as the absence of intestinal ulcerations among patients with ulcerations at baseline (Hanauer *et al.* 2002; Colombel *et al.* 2010), and has been used as primary end point in the adalimumab EXTEND trial (Rutgeerts *et al.* 2012). The major limitation of using mucosal healing as an outcome measure is that partial resolution of ulcers is not considered (Levesque *et al.* 2015). However, a post-hoc analysis of the SONIC trial showed that both a 50% decrease in endoscopic activity indices (CDEIS and SES-CD) and mucosal healing are the most robust predictors of steroid-free remission at week 50 from the treatment start (Ferrante *et al.* 2013).

The Lémann index

In order to evaluate disease progression in CD, the Lémann index has been developed based on a comprehensive evaluation of cumulative structural intestinal damage, including strictures, fistulae, and surgical resections, using clinical, upper and lower endoscopic and abdominal and pelvic MRI assessment (Pariente *et al.* 2015). Using the Lémann index it has been reported that 62% of patients have substantial intestinal damage from 2 to 10 years from the diagnosis, and that high Lémann index scores at the first evaluation, time, persistent clinical activity, and intestinal resection are associated with damage (Gilletta *et al.* 2015).

1.3.2.5 Treatment

The key points in the therapeutic management of adult CD patients are the induction of remission during active disease and the maintenance of remission in order to prevent disease progression and development of complications. One of the most important measures to adopt in CD is to strongly recommend the patient to stop smoking, as this is associated with a 65% reduction in relapse risk

(Johnson *et al.* 2005). Treatment options for active CD include nutritional support and nutritional therapy, an expanding range of anti-inflammatory drugs and immunomodulators with considerable variability in specificity and targets, and surgery (Mowat *et al.* 2011).

Nutritional support and nutritional therapy

Malnutrition is a common feature in CD, and particular attention should be paid to the correction of micronutrient deficiencies such as calcium, vitamin B12, folate, iron and zinc (Mowat *et al.* 2011). Total parenteral nutrition may be required in CD patients who develop short bowel syndrome after surgical resection of affected intestine, or in the perioperative care of patients with significant weight loss (Mowat *et al.* 2011). Exclusive enteral nutrition is effective in inducing remission both in small bowel and colonic disease in 60-80% children with active CD, with no difference between elemental and polymeric diet. Liquid feeding has the great advantages of ensuring optimal growth and avoiding toxicity of corticosteroids in children with CD (Zachos *et al.* 2007; Mowat *et al.* 2011). A retrospective study showed that the combination of partial enteral nutrition and exclusion diet induces clinical remission in children and young adults with mildly and moderately active luminal CD (Sigall-Boneh *et al.* 2014). However, exclusive enteral nutrition is more effective than partial enteral nutrition in reducing mucosal inflammation in children with active CD (Lee *et al.* 2015b). It has been proposed that dietary intervention could be used as an adjunct therapy in specific subsets of patients in order to reduce the risk of relapse or the degree of immunosuppression necessary to control the disease (Lee *et al.* 2015a).

Medical therapy

The majority of patients with active CD are treated with anti-inflammatory drugs such as corticosteroids, immunosuppressive agents such as thiopurines and methotrexate, and biologic drugs with a specific target such as anti-TNF- α and

anti-integrin agents (Danese 2012). While 5-aminosalicylates have limited efficacy in CD, corticosteroids such as prednisone have been used as first-line therapy in most patients with active CD (Dignass *et al.* 2010). Thiopurines, which include azathioprine and 6-mercaptopurine, are effective in inducing and maintaining remission and are associated with a 40% reduction of surgical resections in CD, however they have a slow onset of action and patients must be carefully monitored for the risk of hepatotoxicity, pancreatitis and leucopaenia (Chatu *et al.* 2014; Goel *et al.* 2015). Methotrexate is very appropriate in CD patients with arthropathy, as both disorders can benefit from this drug (Baumgart *et al.* 2012). Patients with perianal CD benefit from treatment courses with antibiotics such as metronidazole and ciprofloxacin, which are also used for the treatment of complications such as abscesses (Mowat *et al.* 2011).

The management of active CD has been revolutionised by anti-TNF- α agents, such as the chimaeric murine-human monoclonal antibody infliximab and the fully human monoclonal antibody adalimumab, which can induce both clinical remission and mucosal healing in luminal CD, as shown in the SONIC and EXTEND trials, and are also effective in fistulising CD (Colombel *et al.* 2010; Mowat *et al.* 2011; Rutgeerts *et al.* 2012). It has been shown that dose adjustment of infliximab based on serum trough level measurement is associated with fewer flares of CD during the course of treatment compared to clinically-based dosing (Vande Casteele *et al.* 2015). In particular, the Authors recommend continued concentration-based infliximab dosing, aiming for serum trough levels ≥ 3 and ≤ 7 $\mu\text{g/ml}$, which maximise response to treatment and minimise costs and potential side effects (Vande Casteele *et al.* 2015). A relevant limitation in the use of anti-TNF- α agents resides in their high cost. However, after the patent for infliximab has expired, the biosimilar infliximab has been developed and approved in Europe, UK and US. The cheaper price of biosimilars will enable access to anti-TNF- α treatment to a wider population of patients, and this may have a substantial impact on therapeutic paradigms in CD (Schreiber 2015).

Other biologic agents with alternative mechanisms of action have also been successful in randomised clinical trials in CD. In particular, both ustekinumab, a fully human monoclonal antibody specific for IL-12 and IL-23 p40 subunit, and vedolizumab, an anti- $\alpha_4\beta_7$ integrin monoclonal antibody, have shown clinical efficacy in CD (Sandborn *et al.* 2012b; Sandborn *et al.* 2013b; Wils *et al.* 2016). Finally, mongersen, an oral antisense oligonucleotide targeting the inhibitor of TGF- β signalling Smad7, has been shown to induce significantly higher clinical response and remission rates compared with placebo (Monteleone *et al.* 2015).

Surgical options

When the outcome of medical therapy is not satisfactory and in case of complications such as internal or complicated perianal fistulae and fibrotic strictures, surgical management of CD should be considered. However, surgery is not curative in CD, and, rather than extensive destructive intestinal resections, the current trend is to resect or treat the segment responsible for the main symptoms and leave the rest of the affected intestine in place (Dignass *et al.* 2010). The cumulative incidence of abdominal surgery in CD is 33% at 5 years and 47% at 10 years from the diagnosis (Frolkis *et al.* 2013). When the extent of CD lesions is very limited, such as in ileocaecal CD, surgical resection of the affected tract is a valid option. Typically, fibrotic strictures causing intestinal occlusive symptoms in the jejunum and ileum may be treated with stricturoplasty. Conversely, stricturoplasty in the colon is not recommended (Dignass *et al.* 2010). Seton drainage is commonly performed in perianal CD, whereas advancement flaps and fistula plugs may be appropriate for persistent or complex fistulae in combination with medical treatment.

Unfortunately, most patients develop recurrence of CD lesions in the neo-terminal ileum or at the anastomosis after surgical intestinal resection, however smoking cessation substantially reduces post-operative relapse (Mowat *et al.* 2011). Macroscopic recurrence of lesions after surgery in CD can be graded

endoscopically according to the Rutgeerts' score (Rutgeerts *et al.* 1990). In particular, 0 indicates absence of lesions; 1: less than 5 aphthous lesions; 2: more than 5 aphthous lesions with normal mucosa between the lesions, or skip areas of larger lesions, or lesions confined to the ileo-colonic anastomotic lining (< 1cm); 3: diffuse aphthous ileitis with diffusely inflamed mucosa; 4: diffuse ileal inflammation with larger ulcers, nodules, or narrowing. Hyperaemia and oedema alone are not considered as signs of recurrence (Rutgeerts *et al.* 1990). The POCER trial explored the optimal approach to prevent post-operative disease recurrence, and established that, after surgery, a strategy based on treatment with thiopurine or adalimumab in case of high clinical risk of recurrence (smoking and/or penetrating disease and/or previous resection) followed by colonoscopy after 6 months and treatment step-up in case of recurrence of lesions is more effective than conventional step-up management for the prevention of post-operative recurrence in CD (De Cruz *et al.* 2015).

Management strategies in CD – “Step-up”, “top down” and “treat-to-target”

The traditional way to treat active CD has been following the so-called “step-up” approach, with step-wise therapy intensification and change in drug class according to the response to treatment and to the frequency and severity of subsequent relapses (Fig. 1.3A). However, the decision to escalate treatment is often based on clinical assessment, and it is well known that there is poor correlation between clinical and endoscopic activity (Peyrin-Biroulet *et al.* 2011). Moreover, as a result of the incremental step-up approach, the decision to start immunosuppression or biologic therapy in the most aggressive cases of CD is delayed, with potential negative consequences on disease progression. It has been demonstrated that early combined immunosuppression with infliximab and azathioprine has superior efficacy compared with step-up therapy and to monotherapy with either infliximab or thiopurines in CD, and leads to sustained, steroid-free remission (D’Haens *et al.* 2008; Colombel *et al.* 2010).

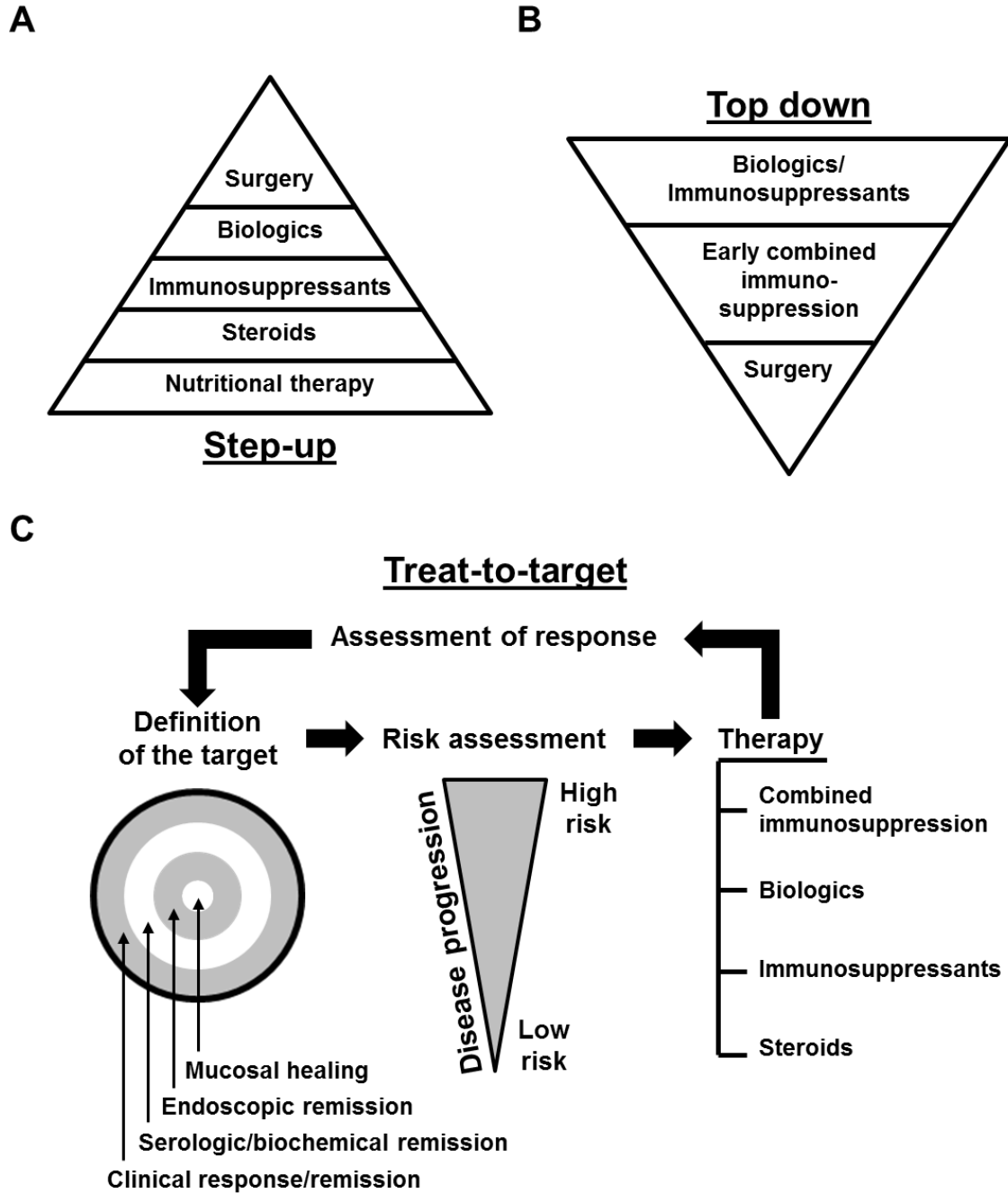


Figure 1.3

Figure 1.3. Possible treatment strategies in CD. (A) The “step-up” treatment approach has been traditionally followed in order to induce remission in patients with active CD. Treatment is decided based on disease activity, and escalation to a more aggressive and/or effective treatment is dictated by non-responsiveness

or subsequent relapse. **(B)** Following the observation that early combined immunosuppression with infliximab and azathioprine has superior long-term efficacy compared with step-up therapy and to monotherapy with either infliximab or thiopurines, the alternative “top down” approach has been proposed for CD, with the idea that using a combination of biologic and immunosuppressant early in the disease may reduce the need for hospitalisation and surgery. **(C)** The “treat-to-target” approach in CD is based on the definition of a specific therapeutic target and the subsequent selection of the most appropriate treatment depending on the risk of disease progression. The achievement of the selected target is evaluated by regularly assessing response to treatment, and therapy adjustment is performed accordingly. In particular, assessment of response can be performed by clinical examination in case patient-reported outcomes such as clinical response and remission have been chosen as targets; by blood and stool tests in order to evaluate serologic (CRP) and biochemical (faecal calprotectin) remission; by ileocolonoscopy to assess endoscopic remission and mucosal healing. (Original Figure).

Therefore, the alternative “top down” approach has been proposed, with the idea that starting with a combination of biologic and immunosuppressant and de-escalate if possible may improve disease course and reduce the need for hospitalisation and surgery in CD (Fig. 1.3B). However, CD is a heterogeneous disease, and the top down strategy may lead to overtreatment in a substantial proportion of patients. One way to avoid this is to stratify CD patients at diagnosis according to the presence of risk factors for disease progression. In particular, age < 40 years at diagnosis, rectal or small bowel lesions, perianal disease, deep ulcerations and need for steroids to treat the first flare are all associated with disabling disease course in CD (Beaugerie *et al.* 2012). In the REACT trial, CD patients who failed to achieve clinical remission with a course of steroids were randomised to receive either conventional step-up therapy or early combined immunosuppression (Khanna *et al.* 2015). The rate of steroid-free clinical remission at 12 months did not differ between the two groups, however at 24 months patients assigned to early combined immunosuppression had significantly lower rates of intestinal resections and CD complications such as fistulae and strictures, supporting the concept that management based on risk stratification may improve natural history in CD.

Clinical experience in rheumatoid arthritis has shown that management based on the regular assessment of pre-defined therapeutic targets with objective measures and consequent treatment adjustment leads to optimal outcomes for patients (Schipper *et al.* 2010). These considerations prompted the formulation of the “treat-to-target” approach for CD, in which the desired target is specified before starting the treatment, and the intervention is decided upon careful risk assessment, with a view to start early immunosuppression in high-risk patients (Fig. 1.3C) (Bouguen *et al.* 2015). Response to treatment is then assessed regularly until the target is reached, and drug therapy is adjusted accordingly. Consensus-based recommendations of therapeutic targets for CD have been formulated, and include both the patient-reported outcome of clinical remission, defined as resolution of abdominal pain and diarrhea/altered bowel habit, and endoscopic remission, defined as resolution of ulceration at ileocolonoscopy, or resolution of findings of inflammation on cross-sectional imaging in patients who cannot be adequately assessed with ileocolonoscopy, whereas serologic (CRP)/biochemical (faecal calprotectin) remission has been considered as an adjunctive target (Peyrin-Biroulet *et al.* 2015). It has been suggested that in a clinical setting the “treat-to-target” approach may be realistically feasible by performing a first endoscopic assessment every six months after the treatment start until disappearance of intestinal ulcers, then every 1-2 years thereafter (Bouguen *et al.* 2015).

1.3.3 UC

1.3.3.1 Epidemiology

The annual incidence of UC is up to 19.2 per 100,000 inhabitants in North America and up to 24.3 per 100,000 inhabitants in Europe, depending on different geographical areas (Ananthakrishnan 2015). In the UK, UC affects at least 146,000 patients, with a prevalence of approximately 240 in 100,000 inhabitants (NICE 2013; www.crohnsandcolitis.org.uk). UC occurs at approximately the same rate in men and women (www.crohnsandcolitis.org.uk). The main peak of incidence in UC is between the ages of 15 and 25, with a second, smaller peak between 55 and 65 years, although this latter has not been conclusively demonstrated (NICE 2013).

1.3.3.2 Diagnosis

The diagnosis of UC is based on the presence of clinical symptoms confirmed by endoscopic and histologic findings (Dignass *et al.* 2012a).

Clinical manifestations and laboratory investigations

Typical manifestations of UC are rectal bleeding and diarrhoea, urgency and tenesmus. In severe cases, fever and tachycardia may be present. In a subject with suspect UC, initial laboratory tests should include full blood count, ESR and/or CRP, urea and electrolytes, liver function tests and ferritin (Dignass *et al.* 2012a). Infectious colitis needs to be ruled out by performing stool cultures and by testing for *Clostridium difficile* toxin A and B (Dignass *et al.* 2012a). Faecal calprotectin is an accurate marker of colonic inflammation, it is more sensitive than CRP as a biomarker of endoscopic inflammation, and it may be useful in monitoring disease activity or response to therapy (Lewis *et al.* 2011; Mosli *et al.* 2015). Serum perinuclear anti-neutrophil cytoplasmic antibodies (pANCA) are

present in up to 65% UC patients and in less than 10% CD patients, however their use in clinical practice is currently not recommended (Dignass *et al.* 2012a).

Endoscopic and histologic findings

Pancolonoscopy with retrograde ileoscopy and segmental biopsies including the rectum are the preferred procedures to establish the diagnosis of UC (Dignass *et al.* 2012a). In UC, colonoscopy reveals signs of inflammation such as mucosal erythema and hyperemia on friable mucosa and multiple ulcerations, however no endoscopic feature is specific for UC (Dignass *et al.* 2012a). The continuous damage-regeneration process occurring in inflamed areas leads to the growth of pseudopolyps. The most indicative endoscopic signs of UC (as opposed to CD) are rectal involvement with a caudo-cranial extension and a continuous distribution (Dignass *et al.* 2012a). Disease extent should be assessed at diagnosis, because of prognostic and therapeutic implications.

A reliable diagnosis of UC requires the histologic analysis of at least two biopsies from at least five intestinal segments, including the ileum and the rectum (Dignass *et al.* 2012a). Microscopically, crypt abscesses, crypt architectural distortion with branching and atrophy, mucin depletion, and basal cell plasmacytosis (presence of plasma cells around or below the crypts) are typical features of UC (Dignass *et al.* 2012a; Feakins 2013).

Complications

UC can be associated with primary sclerosing cholangitis, a chronic cholestatic liver disease characterised by a fibrosing inflammatory destruction of intrahepatic and extrahepatic bile ducts, which occurs in up to 3% of patients (Rothfuss *et al.* 2006). The presence of primary sclerosing cholangitis increases the risk of both cholangiocarcinoma and colo-rectal cancer (CRC) (Van Assche *et al.* 2013).

Importantly, the risk of thromboembolism is doubled in patients with UC compared with control subjects (Van Assche *et al.* 2013).

Patients with UC have a higher risk of CRC compared with the general population, and this is correlated with disease extent and duration (Van Assche *et al.* 2013). Indeed, it has been reported that the cumulative probability of CRC development is estimated at 2%, 8% and 18% respectively after 10, 20 and 30 years of disease duration (Lakatos *et al.* 2008). The presence of severe and persistent disease activity, colonic pseudopolyps, primary sclerosing cholangitis and positive family history for CRC confer an additional risk for CRC in UC patients (Baars *et al.* 2011; Van Assche *et al.* 2013). Colon carcinogenesis in UC is characterised by a multi-step process from low-grade dysplasia (with stratified, bottom-placed, elongated, hyperchromatic nuclei and increased number of mitoses) to high-grade dysplasia (marked nuclear atypia, loss of cell polarisation, branched crypts) and finally to adenocarcinoma (Triantafillidis *et al.* 2009). Colonic stenosis seen endoscopically in UC should raise the suspicion of CRC, and prompt radiologic imaging in case intubation is not possible (Dignass *et al.* 2012a).

1.3.3.3 Classification

According to the Montreal classification (Silverberg *et al.* 2005; Satsangi *et al.* 2006), UC can be classified into different clinical phenotypes depending on the maximal extent of intestinal inflammation observed at colonoscopy (Montreal E-category). In particular, E1 indicates proctitis, when only the rectum is involved; E2, left-sided colitis, extending up to splenic flexure; E3, more extensive disease, which can be defined as pancolitis when all the colon is involved (Silverberg *et al.* 2005). At diagnosis, UC is limited to the rectum in approximately one third of patients, it extends up to the splenic flexure in another third, and proximally to the splenic flexure in another third, with 20-25% of patients having pancolitis. Disease extent tends to change over the course of UC: distal colitis progresses

proximally in about 50% of patients, whereas in more than 2/3 of pancolitic patients the extent of colonic involvement decreases over time (Cosnes *et al.* 2011). Disease extent is an important predictor of colectomy and CRC (Ordás *et al.* 2012).

1.3.3.4 Disease activity indices

Since the first randomised controlled trial in UC (Truelove *et al.* 1955), several indices of disease activity based on clinical, laboratory, endoscopic or histologic variables have been developed in clinical trial design in UC. However, none of them has been formally and completely validated according to the guidance of the US Food and Drug Administration for the development of patient-reported or clinician-reported outcomes (Levesque *et al.* 2015).

Truelove and Witts' criteria

The Truelove and Witts' criteria (Truelove *et al.* 1955), which are derived from the Truelove and Witts Severity Index (Truelove *et al.* 1955), are the only disease activity index to be used routinely in clinical practice in UC, and define acute severe UC (ASUC) as the passage of six or more stools with blood per day associated with one or more signs of systemic toxicity (temperature > 37.8°C; tachycardia > 90 bpm; haemoglobin < 105 g/L; ESR > 30 mm/h; CRP > 30 mg/L). In particular, the Truelove and Witts' criteria are used to identify UC patients in need of hospital admission and intensive treatment (Macken *et al.* 2015).

Powell-Tuck Index, Clinical Activity Index (CAI) and Lichtiger score

Other clinical indices of disease activity in UC include the Powell-Tuck Index (also known as St Mark's Index) (Powell-Tuck *et al.* 1978), which considers 10 gastrointestinal and systemic clinical variables and was subsequently modified to include macroscopic appearance at sigmoidoscopy (Powell-Tuck *et al.* 1982),

and the CAI (or Rachmilewitz Index) (Rachmilewitz 1989). This latter is composed of seven clinical variables (number of stools, blood in stools, investigator's global assessment of symptomatic state, abdominal pain or cramps, temperature due to colitis, extraintestinal manifestations, and laboratory findings) and ranges from 0 to 29 points, with remission being defined as a score ≤ 4 .

In the first pilot trial on the use of intravenous ciclosporin in ASUC, a modified Truelove and Witts Severity Index, also known as the Lichtiger score (Lichtiger *et al.* 1994), was used to assess response to treatment. The Lichtiger Score includes seven clinical variables and ranges from 0 to 21 points, ASUC is defined as a score > 10 points, and remission has been defined as a score ≤ 3 points (D'Haens *et al.* 2007; Laharie *et al.* 2012).

Endoscopic or composite clinical and endoscopic disease activity indices

In addition to clinical indices, multiple endoscopic or composite clinical and endoscopic indices of disease activity have been developed in UC.

The Baron Score was developed to assess interobserver variability in describing rectosigmoid mucosa using a rigid proctoscope (Baron *et al.* 1964), and considered eight endoscopic descriptors such as friability, vascular pattern and spontaneous bleeding. The Modified Baron Score removed qualitative assessment of different levels of bleeding (moderately, severely), established five grades by categorical variables (0, normal; 1, granular; 2, friable; 3, bleeding; 4, ulcerated), and defined endoscopic response as an improvement of 2 grades and endoscopic remission as a score of 0 (Feagan *et al.* 2005).

The Mayo Score (also known as Disease Activity Index) is a composite index that includes three clinical variables (stool frequency, rectal bleeding, physician's global assessment) and endoscopic severity at flexible sigmoidoscopy, each one ranging from 0 to 3 (Schroeder *et al.* 1987). Complete remission is defined as a

subscore of 0 in all variables. The main limitation of the Mayo Score is that it assimilates symptoms and endoscopic variables that are not logically combinable and are difficult to appropriately weight, with consequent detrimental effect on measurement variability and statistical efficiency. A possible approach to overcome this limitation has been to independently assess endoscopic inflammation and clinical variables of the Mayo Score, using a 3-item (stool frequency, rectal bleeding and physician's global assessment) or a 2-item (stool frequency, rectal bleeding) clinical Mayo score, and an endoscopic Mayo subscore (Levesque *et al.* 2015). In particular, in the endoscopic Mayo subscore, 0 indicates normal or inactive disease; 1, mild disease with erythema, decreased vascular pattern, mild friability; 2, moderate disease with marked erythema, absent vascular pattern, friability, erosions; 3, severe disease with spontaneous bleeding and/or ulceration.

Mucosal healing in UC has been classically defined as an endoscopic Mayo subscore 0 or 1 (Colombel *et al.* 2011). It has been shown that UC patients with an endoscopic Mayo subscore of 1 have a significantly higher risk of clinical relapse (defined as the need for therapy to induce remission, any treatment escalation, hospitalisation or colectomy) in the following 6-12 months compared with those with subscore of 0 (Barreiro-de Acosta *et al.* 2016; Boal Carvalho *et al.* 2016), suggesting that the definition of mucosal healing should be limited to endoscopic Mayo subscore of 0.

In contrast to the empirically developed endoscopic scoring systems described so far, the Ulcerative Colitis Endoscopic Index of Severity (UCEIS) has been developed upon the analysis of 630 assessments of 60 endoscopic videos by 10 specialists (Travis *et al.* 2012b). Initially defined as an 11-point score, the UCEIS has been subsequently simplified to an 8-point score with the following variables: erosions and ulcers (score 0-3), vascular pattern (score 0-2) and bleeding (score 0-3), and has shown high correlation with overall endoscopic assessment of severity (Travis *et al.* 2013).

Histologic disease activity indices

Several histologic indices of activity have also been developed in UC, in most cases empirically (Mosli *et al.* 2014). The first one to be described was the 3-grade (mild, moderate and severe) Truelove and Richards Index (Truelove *et al.* 1956), which was used in a study showing that microscopic inflammation can be present in UC patients with macroscopically normal mucosa and predicts a higher risk of relapse.

Originally developed as a 5-point scale evaluating the degree of chronic inflammatory infiltrate and tissue destruction (Riley *et al.* 1988), the Riley Score was subsequently designed as a 4-point scale (normal, mild, moderate, severe) assessment of six independent variables (neutrophil infiltrate, crypt abscesses, mucin depletion, surface epithelial integrity, chronic inflammatory cell infiltrate and crypt architectural irregularities) (Riley *et al.* 1991). Unlike endoscopic evidence of erythema, histologic inflammation, graded using the Riley Score, was shown to predict relapse of UC in patients with clinically quiescent disease and normal or erythematous rectal mucosa (Riley *et al.* 1991). The Modified Riley Score ranked the degree of inflammation hierarchically according to the presence of neutrophils in the epithelium (mild), neutrophil infiltration in the lamina propria (moderate), erosions or ulceration (severe) (Feagan *et al.* 2005).

Finally, the more comprehensive Geboes Score incorporated a number of previously reported variables, including architectural changes, epithelial and lamina propria neutrophils, crypt destruction, erosion or ulceration (Geboes *et al.* 2000). The Geboes Score showed a good interobserver agreement between pathologists for biopsies collected from endoscopically inflamed mucosa of UC patients (Geboes *et al.* 2000). Both the Modified Riley Score and the Geboes Score show a highly significant correlation with the Mayo endoscopic subscore in case of both severely active and inactive disease, however there is high diversity in mildly and moderately active disease (Lemmens *et al.* 2013).

1.3.3.5 Treatment

The treatment goals in UC are to induce remission during a disease flare and to maintain remission in order to prevent complications and colectomy. Several anti-inflammatory agents, immunosuppressants and immunomodulators may be used in active UC, however the choice of a specific agent and its way of administration depend on the different features of disease in a particular patient. The key aspects to consider when making therapeutic decisions in active UC are disease extent and severity, and outcomes of previous treatments (Mowat *et al.* 2011; Dignass *et al.* 2012b).

Medical therapy for the induction of remission

5-aminosalicylates such as mesalazine are the first-line therapy for patients with mild to moderate UC and can be administered topically (suppositories in case of proctitis or enemas in case of more extensive disease) and/or orally (in case of left-sided colitis or pancolitis), depending on disease extent (Dignass *et al.* 2012b). Addition oral mesalazine or topical steroids to topical mesalazine should be considered for escalation of treatment in proctitis (Dignass *et al.* 2012b). Topical steroids may be added to topical mesalazine, however they are less effective than mesalazine (Marshall *et al.* 2010). In left-sided colitis and more extensive mild to moderate UC, the first-line treatment is combined oral and topical mesalazine (Dignass *et al.* 2012b).

If mesalazine fails to induce improvement in 2-4 weeks, oral corticosteroids should be started (Mowat *et al.* 2011; Dignass *et al.* 2012b; Ordás *et al.* 2012). While 70% patients respond to steroids, the remaining 30% do not respond or develop steroid-dependence and should be switched to thiopurines and/or anti-TNF- α agents (Ordás *et al.* 2012). Azathioprine is moderately effective in inducing clinical and endoscopic remission in steroid-dependent UC (Ardizzone

et al. 2006; Leung *et al.* 2008; Louis *et al.* 2014). Both infliximab and adalimumab have shown good ability to induce mucosal healing, defined as endoscopic Mayo subscore ≤ 1 , in patients with moderate to severe chronically active UC (Rutgeerts *et al.* 2005; Reinisch *et al.* 2013). It has been shown that combination of infliximab and azathioprine is superior to either drug as monotherapy in moderate to severe chronically active UC (Panaccione *et al.* 2014), however further studies are needed before combination therapy can be conclusively recommended in UC, and an individualised approach appears currently preferable (Lee *et al.* 2014).

Other therapeutic agents or interventions have been tested, and in certain cases approved, for the treatment of active UC. In particular, the fully human monoclonal anti-TNF- α antibody golimumab was effective in inducing clinical remission and mucosal healing (endoscopic Mayo subscore ≤ 1) in patients with moderately to severely active UC (Sandborn *et al.* 2014). In the UK, golimumab has been recommended as an alternative to infliximab and adalimumab for UC patients with moderately or severely active disease who did not respond to or could not tolerate corticosteroids or thiopurines (NICE 2015a). The anti- $\alpha_4\beta_7$ integrin humanised monoclonal antibody vedolizumab is significantly more effective than placebo in inducing clinical remission and mucosal healing (endoscopic Mayo subscore ≤ 1) in UC (Feagan *et al.* 2013), and in the UK vedolizumab has been recommended for moderately to severely active UC patients who failed therapy with conventional therapy or with an anti-TNF- α agent (NICE 2015b). The oral Janus kinase (JAK) inhibitor tofacitinib induced clinical remission and mucosal healing (endoscopic Mayo subscore 0) in a dose-dependent manner in moderately or severely active UC (Sandborn *et al.* 2012c), however its safety profile needs to be carefully evaluated before recommending its use in clinical practice to treat UC (Peyrin-Biroulet *et al.* 2013).

Finally, faecal microbiota transplantation has given contrasting results in two randomised controlled clinical trials in mild to moderate UC, possibly due to

different route and frequency of administration (Moayyedi *et al.* 2015; Rossen *et al.* 2015). In particular, duodenal infusion of donor faeces, which is significantly more effective than vancomycin for recurrent *C. difficile* infection (van Nood *et al.* 2013), had no significant effect on mild to moderate UC (Rossen *et al.* 2015). Conversely, weekly faecal microbiota transplantation via retention enema for six weeks induced endoscopic remission in a significantly higher proportion than placebo in patients with mild to moderate UC (Moayyedi *et al.* 2015), however this was achieved only in 24% of randomised patients.

Management of ASUC

The management of ASUC follows a different algorithm from chronically active UC. ASUC is a medical emergency which requires admission and intravenous hydrocortisone administration due to the high risk of colectomy (Macken *et al.* 2015). A plain abdomen radiograph is required to exclude the presence of toxic megacolon or perforation, and an unprepared flexible sigmoidoscopy with biopsies may be important for the diagnosis and management, however it should not delay the treatment start (Macken *et al.* 2015). Moreover, patients admitted for ASUC require venous thromboembolism prophylaxis and fluid/electrolyte balance correction (Macken *et al.* 2015). Nutritional support is important in malnourished patients with ASUC (Dignass *et al.* 2012b). There is not sufficient evidence to recommend total parenteral nutrition with bowel rest in ASUC, whereas in this condition enteral nutrition is most appropriate and associated with significantly fewer complications than parenteral nutrition (Van Gossum *et al.* 2009).

Intravenous hydrocortisone induces remission in 70% patients with ASUC. However, failure of steroids by day 3, defined according to the Oxford criteria as stool frequency > 8/day, or stool frequency 3-8/day and a CRP > 45mg/L, predicts an 85% likelihood of requiring a colectomy, and prompts escalation to rescue therapy with infliximab or ciclosporin or tacrolimus, or referral for

colectomy (Dignass *et al.* 2012b; Macken *et al.* 2015). It has been shown that ciclosporin has equivalent efficacy to infliximab in steroid-resistant ASUC, with no significant difference between the two treatments in the rate or time to colectomy (Laharie *et al.* 2012). Colectomy should be considered in patients who do not respond to rescue therapy within 7 days from initiation (Macken *et al.* 2015). A retrospective study showed that patients with ASUC who were given an accelerated induction with three doses of infliximab within a median of 24 days had a significantly lower rate of colectomy at 3 months as compared with the standard 0, 2 and 6 week induction regimen (Gibson *et al.* 2015).

Approximately 5% patients with ASUC develop toxic megacolon, which can be defined as a total or segmental non-obstructive dilatation of the colon (≥ 5.5 cm) on plain abdominal X-ray, associated with systemic toxicity (Gan *et al.* 2003). Hypomagnesaemia, hypokalaemia and anti-diarroheal medications are risk factors for toxic megacolon (Dignass *et al.* 2012b). In case of toxic megacolon, in addition to intravenous hydrocortisone, empirical treatment with oral vancomycin should be considered until stool is confirmed negative for *C. difficile* toxin, and surgical opinion should be sought, as an early colectomy may be necessary (Dignass *et al.* 2012b).

Medical therapy for the maintenance of remission

Once active UC has been successfully treated, the therapeutic goal is to maintain steroid-free clinical and endoscopic remission. Oral mesalazine is the first-line therapy for maintenance of remission in UC patients (Mowat *et al.* 2011). MMX-mesalazine administered once daily represents a valid alternative to traditional mesalazine and allows better patients' compliance (Yang *et al.* 2011). Azathioprine and 6-mercaptopurine are effective for maintaining remission in UC (Timmer *et al.* 2012), and are recommended as maintenance therapy for patients who are intolerant to 5-aminosalicylates, ASUC patients responding to ciclosporin or tacrolimus for induction of remission, and as steroid-sparing agents

in steroid-dependent UC (Dignass *et al.* 2012b; Goel *et al.* 2015). Anti-TNF- α therapy may also be continued as maintenance therapy in patients who initially respond to treatment (Dignass *et al.* 2012b), however this is not recommended in the UK (Mowat *et al.* 2011).

CRC prevention strategies in UC

Strategies to prevent CRC in UC include long-term treatment with mesalazine, which has an established chemopreventive effect, and colonoscopy surveillance with dye spray (chromoendoscopy) and targeted biopsies (Van Assche *et al.* 2013). It has been shown that prolonged administration of mesalazine can reduce the risk of UC-associated CRC (Velayos *et al.* 2005), whereas maintenance with thiopurines does not have a significant protective effect on the development of CRC (Jess *et al.* 2014). Patients with CD colitis are now considered to have a similar risk of CRC as UC patients.

According to the British Society of Gastroenterology guidelines (Cairns *et al.* 2010), patients with colitis should have a screening chromoendoscopy with targeted biopsies 10 years after diagnosis. The subsequent follow-up is decided based on the endoscopic and histologic findings. In particular, in patients with dysplasia, colectomy should be considered; patients with extensive moderately or severely active colitis, or family history of CRC in first-degree relatives aged < 50 years, or primary sclerosing cholangitis, or previous evidence of dysplasia and declining surgery should undergo yearly endoscopic surveillance; patients with mildly active colitis, or family history of CRC in first-degree relatives aged < 50 years, or pseudopolyps should undergo endoscopic surveillance every 3 years; patients with no evidence of inflammation should undergo surveillance colonoscopy every 5 years (Cairns *et al.* 2010). In the last decade the rate of CRC and colectomy in UC patients has decreased, likely due to surveillance programs and to the more widespread use of chemoprevention with mesalazine (Van Assche *et al.* 2013; Vester-Andersen *et al.* 2014).

Surgical options and pouchitis

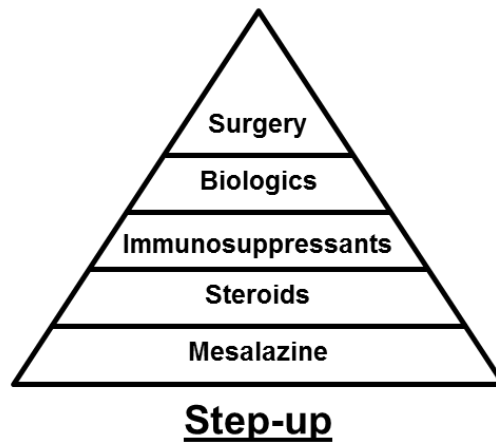
Unlike in CD, surgery can be curative in UC. Emergency surgery is indicated in ASUC and toxic megacolon not responsive to medical management, whereas elective surgery is mainly indicated in patients with dysplasia or CRC, and resistance or contraindication to medical management in active UC (Mowat *et al.* 2011). The most frequently performed surgical technique is total proctocolectomy with ileal J-pouch-anal anastomosis that usually requires a temporary diverting ileostomy (Ordás *et al.* 2012). The cumulative probability of colectomy in UC is 20-30% after 25 years, with disease extent and severity of symptoms at diagnosis being the most accurate predictors (Cosnes *et al.* 2011).

Surgery may be complicated by the development of pouchitis, characterised by high stool frequency and faecal incontinence, which can be acute or chronic if symptoms last less or more than 4 weeks, respectively (Van Assche *et al.* 2013). The first-line treatment of acute pouchitis is metronidazole or ciprofloxacin, whereas chronic pouchitis can be treated with antibiotic combination or budesonide or, in refractory cases, infliximab (Van Assche *et al.* 2013). The best option both for maintaining antibiotic-induced remission and for preventing recurrences of pouchitis is represented by the probiotic preparation VSL#3 (Holubar *et al.* 2010; Van Assche *et al.* 2013).

Management strategies in UC – “Step-up” and “treat-to-target”

The management of chronically active UC still largely relies on a “step-up” approach, with decisions on treatment escalation being guided by outcomes of previous therapy (Mowat *et al.* 2011; Dignass *et al.* 2012b) (Fig. 1.4A).

A



B

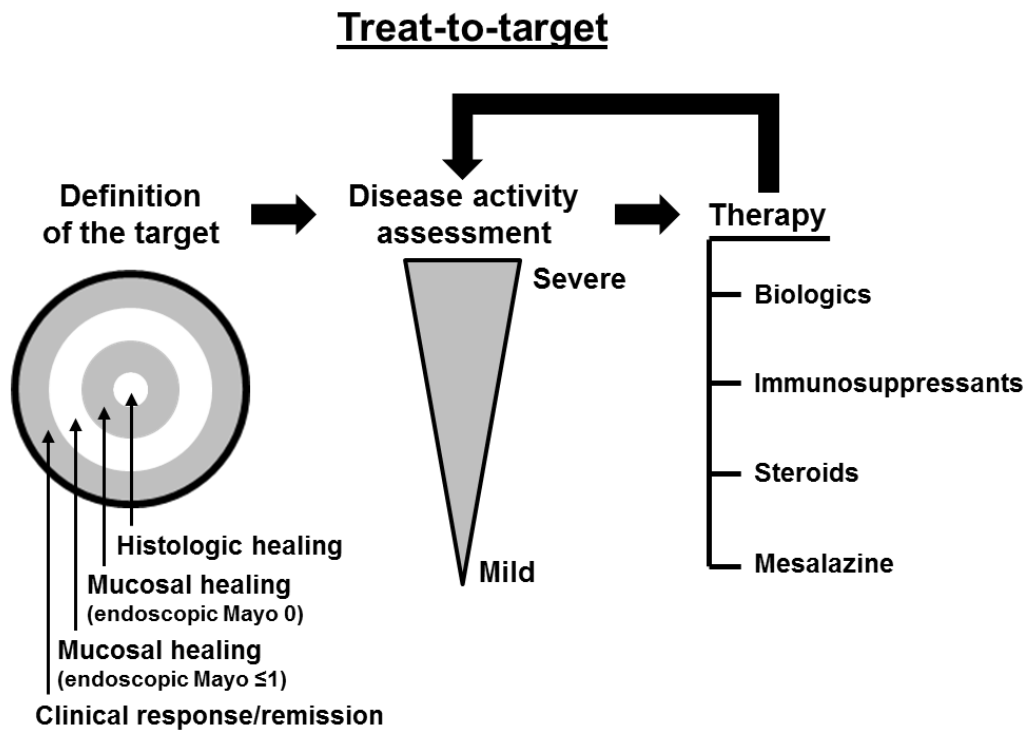


Figure 1.4

Figure 1.4. Possible treatment strategies in chronically active UC. (A) The management of UC in the outpatients setting still largely follows a “step-up” approach, with decisions on treatment escalation being guided by outcomes of previous therapy. (B) The “treat-to-target” approach in UC is based on the definition of a specific therapeutic target and the subsequent selection of the appropriate treatment depending on disease activity. Assessment of disease

activity is repeated at a pre-specified time point, and therapy adjustment is performed accordingly, with the view of leaving the therapeutic target unchanged. (Original Figure).

Conversely, the management of ASUC, based on the application of the Truelove and Witts' criteria to decide which patients need aggressive treatment in order to prevent colectomy, is a good example of patient stratification based on predicted risk of subsequent complications.

Similarly to CD, the “treat-to-target” approach is emerging as a valuable strategy also in the medical management of chronically active UC, however the proposed algorithm in UC is based on disease activity assessment rather than on risk evaluation (Fig. 1.4B) (Levesque *et al.* 2015). The choice of the appropriate target is important in order to prevent long-term negative outcomes in UC, and, as a therapeutic goal, clinical remission has been surpassed by mucosal healing. Failure to achieve mucosal healing after the first course of steroids for newly diagnosed UC predicts a more aggressive disease course at 5 years (Ardizzone *et al.* 2011). Conversely, patients who achieve early mucosal healing (endoscopic Mayo subscore ≤ 1 at week 8 after treatment start) after infliximab induction are less likely to undergo hospitalisation and colectomy at one year compared with those with higher scores (Colombel *et al.* 2011). Histologic healing, which can be defined as the complete resolution of histologic abnormalities, is becoming a potential target not only for assessing disease, but also for predicting disease evolution and consequently for therapeutic decision making in UC (Peyrin-Biroulet *et al.* 2014; Marchal Bressenot *et al.* 2015).

The feasibility of the “treat-to-target” approach in clinical practice was tested in a pilot study in which 60 UC patients were followed-up for 18 months by means of an endoscopic test and therapeutic adjustment within six months from treatment start/change until achievement of the pre-specified targets (mucosal healing – endoscopic Mayo subscore ≤ 1 ; and histologic healing). Most of the patients

underwent two or three endoscopic tests in 18 months, and high cumulative rates of mucosal and histologic healing were observed (60% and 50%, respectively) (Bouguen *et al.* 2014).

1.4 IBD – Aetiopathogenesis

There is evidence that IBD arises as an inappropriate inflammatory reaction to *stimuli* including the intestinal microbiota in genetically susceptible individuals in response to environmental triggers.

1.4.1 Genetic susceptibility in IBD

A number of epidemiologic observations support an important role for genetic susceptibility in the development of IBD. Jewish populations have a higher prevalence compared with non-Jewish population living in the same area (Ananthakrishnan 2015). Additionally, studies on migrant populations showed that, with time, the risk of developing IBD tends to become similar to that of the general population living in the same geographical area. This is more evident particularly from the second generation after the migration, supporting the view that CD and UC arise from a combination of genetic and environmental factors (Cosnes *et al.* 2011). Studies on familial aggregation showed that 5-15% patients with IBD have a positive family history for IBD, and in case both parents are affected, the risk of developing IBD before the age of 30 is 33% (Halme *et al.* 2006). The concordance rate in monozygotic twins is 20-50% for CD and approximately 15% for UC, suggesting that the heritable component is smaller in UC than in CD (Ananthakrishnan 2015).

Several mutations and gene variants, many of which affecting *loci* containing genes involved in microbial handling and immune response, are associated with CD, UC, or both disease (Fig. 1.5) (Lees *et al.* 2011; Jostins *et al.* 2012; McGovern *et al.* 2015). A meta-analysis of genome-wide association studies (GWAS) and ImmunoChip data identified 163 *loci* associated with IBD, which explained 13.6% of CD and 7.5% of UC total disease variances (Jostins *et al.* 2012), and a trans-ethnic analysis of genome-wide and ImmunoChip data identified 38 additional *loci* associated with CD and UC (Liu *et al.* 2015). The

majority of genetic variations are consistently found both in European and in non-European cohorts, and are common to both forms of IBD (Jostins *et al.* 2012; Liu *et al.* 2015). On the other hand, not all *loci* are reported consistently between different GWAS, which underlines the heterogeneity of IBD (Baumgart *et al.* 2007a).

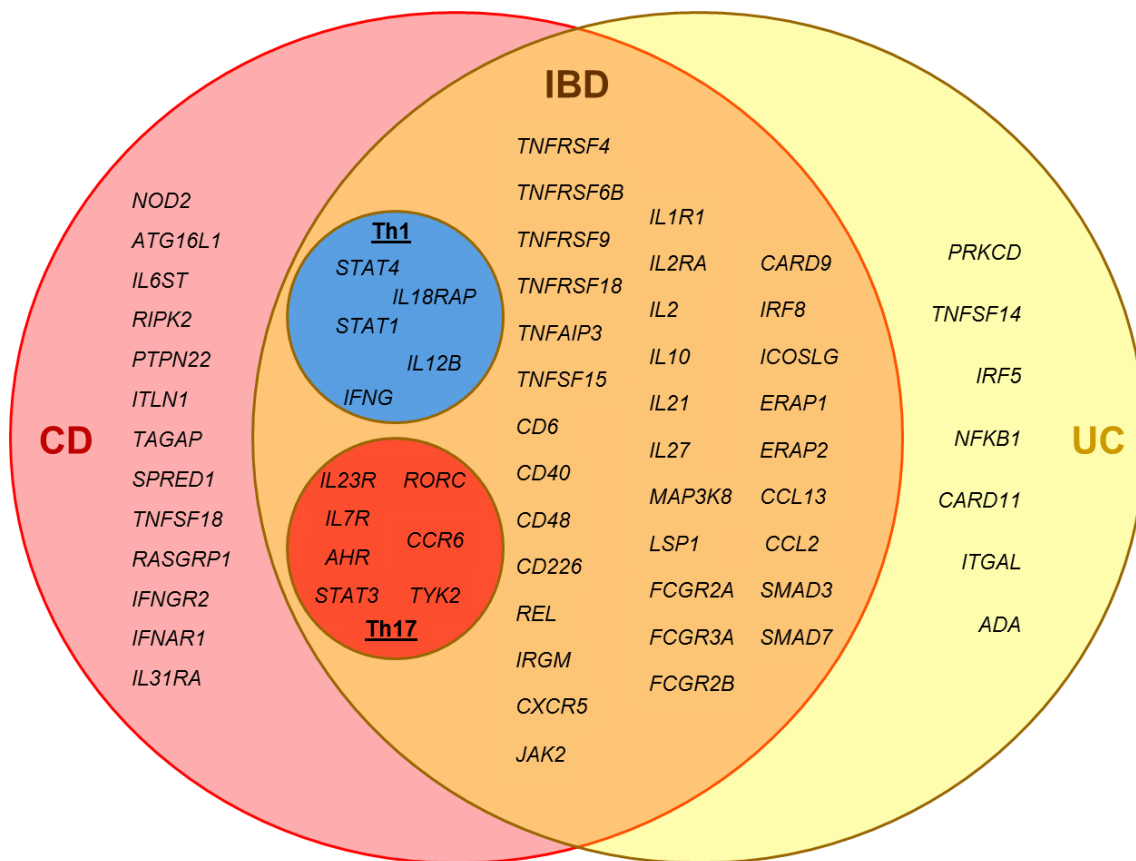


Figure 1.5

Figure 1.5. Heterogeneity of *loci* containing immune response genes associated with IBD. Some of the variants in the listed *loci* are specific for CD, few are specific for UC, and the majority, including several Th1- and Th17-related genes, are common to both forms of IBD. (Adapted from Lees *et al.* 2011).

NOD2 polymorphisms

Three mutations in the gene encoding NOD2, also known as caspase recruitment domain-containing protein 15 (CARD15), on chromosome 16 are associated with a higher risk of CD (Hugot *et al.* 2001; Ogura *et al.* 2001), and studies from France, Germany, UK, and US have shown that up to 40% of patients with CD (compared with 14% of controls) have one or more of these mutations (Cho *et al.* 2007a). Homozygous or compound heterozygous carriage of *NOD2* risk alleles confers a 17-fold increased risk of CD, whereas heterozygous carriage of *NOD2* increases the risk of CD 2.4-fold (Cho *et al.* 2007b). *NOD2* mutations are associated with ileal CD and with a higher likelihood of developing fibrostenosing complications (Abreu *et al.* 2002; Cho *et al.* 2007a).

Several studies have been performed to clarify the functional link between *NOD2* mutations and susceptibility to CD. NOD2 plays an important role in microbial handling, as suggested by the observations that NOD2 stimulation by bacterial MDP induces the release of α -defensins by Paneth cells, activates autophagy in DCs by intracellularly directing bacteria to the autophagosome, and promotes colonic IEC growth and survival (Cruickshank *et al.* 2008; Cooney *et al.* 2010; Tan *et al.* 2015). Mice with targeted *NOD2* mutations show altered TLR signalling, leading to overproduction of pro-inflammatory cytokines and impaired α -defensin release by Paneth cells (Watanabe *et al.* 2004; Kobayashi *et al.* 2005). Overexpression of *NOD2* containing the fs1007 mutation associated with CD in transgenic mouse macrophages results in up-regulated IL-1 β release following MDP stimulation (Maeda *et al.* 2005). Conversely, as compared to control subjects, peripheral blood mononuclear cells (PBMCs) from homozygous *NOD2* mutant CD patients fail to up-regulate IL-8 release upon stimulation with MDP, and do not show up-regulated TNF- α and IL-1 β release upon stimulation with TLR ligands (Van Heel *et al.* 2005). Overall, *NOD2* mutations appear to promote a dysregulated mucosal immune response to microbial components, however the exact mechanisms are unknown, likely due to our incomplete understanding of

the interactions between the host immune system and the intestinal microbiota (Baumgart *et al.* 2007a).

Polymorphisms in autophagy and in IL-23 receptor (IL23R) genes

Variants in the autophagy genes *ATG16L1* and *ATG4B* have been associated with CD (Hampe *et al.* 2007; Liu *et al.* 2015). In particular, the T300A *ATG16L1* polymorphism introduces a caspase-3 or caspase-7 cleavage sequence, resulting in more efficient degradation of the *ATG16L1* allele, with consequent impaired autophagy (Lassen *et al.* 2014; Murthy *et al.* 2014). A study on Scottish population, which has high incidence of CD, the T300A *ATG16L1* was found to be present in 61% adult-onset CD compared with 54% control subjects (Van Limbergen *et al.* 2008). Variants in *IL23R* gene are protective against CD (Duerr *et al.* 2006). In particular, the R381Q polymorphism, which is present in approximately 14% of the European population, is a loss-of-function allele which results in reduced IL-23 signalling (Sarin *et al.* 2011). It has been calculated by a meta-analysis of genetic association studies that the T300A *ATG16L1* and the R381Q *IL23R* variants are associated with 38% increase and a 54% decrease in the risk for CD (Grigoras *et al.* 2015).

Polymorphisms in other immune response and intestinal barrier function genes

Single nucleotide polymorphisms (SNPs) in the *HLA-DRA* gene, encoding for the α -chain of the MHC class II, are strongly associated with an increased risk of UC (McGovern *et al.* 2015). Variants in the gene encoding for IFN regulatory factor (IRF)5, which is a susceptibility gene for rheumatoid arthritis and systemic lupus erythematosus, and whose product induces cytokines such as IL-6, IL-12 and TNF- α via TLR signalling, have been associated with UC (Anderson *et al.* 2011). A highly significant association with UC has also been observed for a common polymorphism in a region on chromosome 1q32 containing the *IL10* gene, with only a very modest association observed in CD (Franke *et al.* 2008). However, it

has been found that the loss-of-function mutations in the *IL-10 receptor* gene results in early-onset IBD with a CD-like phenotype (Glocker *et al.* 2009; Kotlartz *et al.* 2012). GWAS have identified gene variants related to specific pathways of inflammation in IBD (Fig. 1.5). Variants in several genes centrally involved in Th1 pro-inflammatory immune responses, namely *STAT1*, *STAT4*, *IL12B*, *IFNG*, and *IL18RAP*, are associated with increased susceptibility to CD and UC (McGovern *et al.* 2015). Polymorphisms in *AHR*, *STAT3*, *RORC*, and *CCR6*, a group of genes involved in Th17 immune response, are associated with a greater risk for both CD and UC (Duerr *et al.* 2006; McGovern *et al.* 2015).

Finally, mutations in *LAMB1*, *HNF4A*, *GNA12* and *OSMR*, which encode for important molecules for the intestinal barrier function, are associated with a higher risk of IBD (McGovern *et al.* 2015).

1.4.2 Environmental triggers in IBD

There are important evidences that cigarette smoking and appendectomy play a significant role in the aetiopathogenesis of IBD. Moreover, long-term intake of specific dietary factors is likely to influence the susceptibility to IBD, despite relatively few nutrients have been investigated so far, and some of the results have been inconsistent between different studies.

Cigarette smoking

Cigarette smoking is one of the most consistently replicated risk factors for CD (Ananthakrishnan 2015). Smoking is associated with a two-fold increase (odds ratio [OR] 1.76; 95% confidence interval [CI] 1.40-2.22) in the risk of CD and with a similar decrease (OR 0.58; 95% CI 0.45-0.75) in the risk of UC (Mahid *et al.* 2006). Conversely, former smoking is associated with an increased (OR 1.79; 95% CI 1.37-2.34) risk of UC, which remains persistently elevated over 20 years after smoking cessation (Mahid *et al.* 2006; Higuchi *et al.* 2012). Resumption of low dose smoking in a selected group of ex-smokers with treatment-resistant UC may ameliorate signs and symptoms (Calabrese *et al.* 2012). In CD, current smoking is associated with earlier age of onset and more frequent need for immunosuppression in women but not in men, worsens the disease course, favours fistula and stricture formation, and increases the number of flares and post-surgical relapses, whereas in UC current smoking is associated with later age of onset and lower risk of need for immunosuppression in men but not in women (Cosnes *et al.* 2004; van der Heide *et al.* 2009). Less than 40% CD patients are aware of the detrimental effects of smoking (De Bie *et al.* 2015). Current smoking is also associated with a reduced risk of primary sclerosing cholangitis in UC (van der Heide *et al.* 2009), which suggests that the effects of smoking may be systemic and not just limited to the colon.

Several hypotheses have been formulated to explain the reasons behind the complex relationship between smoking and IBD. Nicotine was believed to play an important role due to its ability to increase mucus production and to improve intestinal barrier function in experimental colitis (Cosnes 2004), however trials of nicotine patches or aenemas were not successful (McGrath *et al.* 2004; Ingram *et al.* 2005). PBMCs from smokers with CD, but not UC, show an impaired anti-inflammatory protection against oxidative free radical stress because of lower levels of heat shock protein 70 (Bergeron *et al.* 2012). Polymorphisms in nicotine metabolising genes may influence the association between smoking and IBD (Ananthakrishnan *et al.* 2014b). Moreover, it has been shown that smoking is associated with changes in the intestinal microbiota composition in humans (Biedermann *et al.* 2014).

Appendicectomy

Appendicectomy appears to have a protective effect against the development of UC, since it has been shown to be associated with a 69% reduction in the subsequent risk of UC (Koutroubakis *et al.* 2002). Patients who develop UC after appendicectomy are diagnosed at an older age, are less likely to have recurrent symptoms, and have a reduced need for immunosuppression, however they have a higher risk of primary sclerosing cholangitis (Radford-Smith *et al.* 2002; Gardenbroek *et al.* 2012). As opposed to UC, a higher incidence of CD has been reported following appendicectomy, however it is not clear whether this results from diagnostic problems in patients with undiagnosed CD or whether it may relate to altered microbial handling by the mucosal immune system and failure to develop immune tolerance after appendicectomy (Baumgart *et al.* 2007a; Kaplan *et al.* 2008). The use of oral contraceptive agents is associated with an increased risk of CD, however this effect tends to disappear after suspension (Khalili *et al.* 2013).

Dietary factors

Currently, nutrients which have been investigated in prospective studies in IBD include polyunsaturated fatty acids (PUFAs), vitamin D, fibre, and protein. High dietary intake of the n-3 PUFA docosahexaenoic acid is associated to a reduced risk of developing CD (Chan *et al.* 2014). Another prospective study has shown that high intake of dietary long-chain n-3 PUFAs is associated with a reduced risk of UC, whereas high intake of trans-unsaturated fats appears to be associated with an increased risk of UC (Ananthakrishnan *et al.* 2014a). Higher predicted plasma levels of vitamin D, a micronutrient with anti-inflammatory properties, are associated with a reduced risk of developing CD (Ananthakrishnan *et al.* 2012). Interestingly, normalisation of plasma level of vitamin D is associated with a lower risk of surgery and hospitalisation in CD (Ananthakrishnan *et al.* 2013a). Long-term intake of dietary fibre, particularly from fruit, is associated with significantly lower risk of CD, but not UC (Ananthakrishnan *et al.* 2013b). Finally, in the French cohort of the EPIC-IBD study, a positive association between animal, but not vegetable, protein intake and risk of IBD has been reported (Jantchou *et al.* 2010), whereas in the whole European study no association between body mass index and risk of IBD has been reported (Chan *et al.* 2013).

1.4.3 The intestinal microbiota in IBD

There is abundant evidence that the intestinal microbiota plays an important role in the pathogenesis of intestinal inflammation, and this is epitomised by the observation that, despite germ-free mice have increased susceptibility to infections, such as *Salmonella*, *Shigella*, *Listeria* and *Leishmania*, experimental colitis does not develop under germ-free conditions (Sartor 2004).

Effects of intestinal microbiota alterations and constituents on experimental colitis

It has been shown in various experimental models that, in genetically susceptible hosts, changes in the intestinal microbiota may be required for the development of intestinal inflammation. For example, *Tbx21*^{-/-} *Rag2*^{-/-} ulcerative colitis (TRUC) mice, which lack the transcription factor T-bet in the innate immune system and do not develop colitis spontaneously unless infected with the *Proteobacterium* species *Helicobacter typhlonius* (Powell *et al.* 2012). Mice with a mutation in *ATG16L1* develop a dysfunction in Paneth cells and an altered response to DSS colitis resembling CD, which are both dependent on infection with *Norovirus*, and are prevented by broad-spectrum antibiotics (Cadwell *et al.* 2010).

Conversely, a rational selection of Treg-inducing bacterial strains from the stool microbiota of a healthy volunteer has been shown to ameliorate TNBS-induced colitis (Atarashi *et al.* 2013). It has also been demonstrated that *B. fragilis* polysaccharide A, which can undergo processing by DCs and be presented to T cells despite being a polysaccharide, is responsible for protecting *Rag*^{-/-} mice from T cell transfer colitis exacerbated by *H. hepaticus* (Mazmanian *et al.* 2008). *B. fragilis* polysaccharide A appears to exert its effect by promoting the differentiation of IL-10-producing Tregs and by inhibiting Th1 and Th17 response (Mazmanian *et al.* 2008). These observations suggest that targeted modulation of the intestinal microbiota may hold considerable therapeutic potential in IBD.

Alterations of the intestinal microbiota in IBD

There are significant differences in the intestinal commensal microbiota composition between IBD patients and healthy individuals, and IBD patients have a reduced biodiversity of the intestinal microbial community compared to control subjects (Qin *et al.* 2010; Carding *et al.* 2015). *Firmicutes* and *Bacteroidetes* are underrepresented in the intestinal microbiota of CD patients, whereas *Enterobacteriaceae* and *Proteobacteria* are overrepresented (Kostic *et al.* 2014). In particular, the butyrate-producing *Firmicutes* species *Faecalibacterium prausnitzii*, which is less abundant in patients with IBD, appears to have a protective effect, as patients with high levels are less prone to recurrence following surgical resections (Sokol *et al.* 2008).

High frequency of the pathogen *Mycobacterium avium* subspecies *paratuberculosis* has been reported in CD (Feller *et al.* 2007), and presentation of IBD occurs frequently after gastrointestinal infections (García Rodríguez *et al.* 2006). AIEC has been found to preferentially colonise the ileal mucosa in CD patients (Darfeuille-Michaud *et al.* 2004). AIEC reduces levels of ATG16L1 in epithelial cells, and favours invasion both by inhibiting autophagy and by producing the mucin-degrading protease Vat-AIEC (Nguyen *et al.* 2014; Gibold *et al.* 2015). Finally, *C. difficile* infection is associated with a higher risk of mortality and morbidity in IBD patients (Ananthakrishnan *et al.* 2009). Dysbiosis in IBD is not limited to bacterial species. It has been shown that both CD and UC are associated with the expansion of *Caudovirales*, independently from bacterial dysbiosis (Norman *et al.* 2015), and aberrant immune response to indigenous *fungi* influences susceptibility to experimental colitis (Iliev *et al.* 2012).

Evidences suggesting a contributing role of the intestinal microbiota in IBD

Overall, IBD is more prevalent in the distal ileum and colon, consistent with the idea that they reflect aberrant inflammatory responses to the intestinal microbiota

(Knights *et al.* 2013). Remarkable differences in microbial composition between healthy control subjects and CD have been shown in a large cohort of paediatric new-onset and treatment-naïve CD patients, thereby minimising the influence of confounding factors, such as drugs and disease duration, on the intestinal microbiota and highlighting some important changes preceding the start of treatment (Gevers *et al.* 2014; Hall *et al.* 2014). In particular, in CD samples there was abundance of *Pasteurellaceae*, *Veillonellaceae*, *Neisseriaceae*, and *Fusobacteriaceae*, and depletion of *Bacteroidetes*, *Faecalibacterium*, and *Bifidobacterium* (Gevers *et al.* 2014). These differences were only revealed by analysis of mucosal tissue rather than faecal samples, suggesting that bacteria resident on the mucosal surface may be more relevant for disease development than those present in the lumen (Gevers *et al.* 2014; Hall *et al.* 2014). However, it is currently unclear whether changes in intestinal microbial composition observed in IBD contribute to, or rather are a consequence of the mucosal lesions (Carding *et al.* 2015). Moreover, it has been shown in a prospective study that serologic positivity for a combination of antibodies against *E. coli* outer membrane porin and flagellin, ASCA and pANCA predicts the development of IBD in a low-risk population (van Schaik *et al.* 2013).

There is evidence to suggest that perturbation of the intestinal microbiota with antibiotics in early childhood may influence the mucosal immune response and confers high susceptibility to IBD. This is supported by the observations that, compared to 39% of the control subjects, 58% of paediatric patients with IBD received an antibiotic in their first year of life, and that the relative risk of CD in children who previously used antibiotics is 1.84 higher compared to non-users (Shaw *et al.* 2010; Hviid *et al.* 2011). Moreover, multiple antibiotic courses are associated with a higher risk of IBD than one single course (Kronman *et al.* 2012). On the other hand, antibiotics such as metronidazole and ciprofloxacin are effective in perianal disease and decrease the risk of post-operative recurrences in CD, and both probiotics and antibiotics are useful in the treatment of pouchitis (Mowat *et al.* 2011).

1.4.4 Inappropriate inflammatory reaction in IBD intestinal mucosa

Dysregulation of the intestinal immune response in IBD involves several components of the mucosal immune system (Cader *et al.* 2013). This Section focuses on changes in intestinal barrier and epithelial function, on the aberrant mucosal B cell response, on the mucosal T cell response and the relative cytokines involved.

Impaired intestinal barrier and epithelial function in IBD

One of the key initiating mucosal alterations in IBD is a defect in the intestinal barrier function. Multiple mouse models of colitis, such as *IL-10*^{-/-} mice, *TNF*^{ΔARE} mice, and the CD45RB^{hi}-adoptive transfer model, share a component of barrier loss and epithelial damage (Nalle *et al.* 2015). Interestingly, in *IL-10*^{-/-} mice, barrier loss precedes the onset of colitis. Alterations in tight junction composition play an important role in inducing intestinal epithelial barrier dysfunction and in favouring inflammation. It has been observed that mice with constitutively activated MLCK and consequent increased paracellular permeability, when crossed with mice which spontaneously develop colitis, develop accelerated and more severe intestinal inflammation (Su *et al.* 2009). An important consequence of impaired intestinal barrier function is that it allows luminal antigens, including the microbiota, to interact with the components of the mucosal immune system directly and without regulation. It has been shown that, in *IL-10*^{-/-} mice, before the onset of inflammation, the inner colonic mucus layer has a normal thickness but is penetrable to bacteria (Johansson *et al.* 2014). Moreover, it has been observed that conditional claudin 7 knock-out in IECs determines a selective loss of barrier function in the colon, which leads to the absorption of a bacterial chemoattractant for neutrophils, and to the consequent onset of intestinal inflammation (Tanaka *et al.* 2015).

Patients with IBD have increased intestinal permeability, not only in the inflamed mucosa, but also in non-inflamed areas (Söderholm *et al.* 2002; Hedin *et al.* 2012). The defect in the intestinal barrier function in CD has a genetic component, as suggested by the observation that it occurs in healthy relatives of CD patients (May *et al.* 1993; Buhner *et al.* 2006). Moreover, intestinal barrier impairment has been shown to precede the onset of CD in a patient with positive family history (Irvine *et al.* 2000). *Matriptase* transcripts are significantly down-regulated in inflamed colonic mucosa of both CD and UC compared to control subjects, and this may promote the increase in intestinal permeability observed in IBD (Netzel-Arnett *et al.* 2012). Finally, it has been shown that IBD patients in remission with intestinal barrier defect consequent to excessive IEC shedding and have a higher risk of relapse compared to those with an intact barrier (Kiesslich *et al.* 2012).

IECs may contribute to the inflammatory process by releasing pro-inflammatory cytokines, such as IL-15 and IL-8, or by producing smaller amounts of the tolerogenic factor TSLP (Kaser *et al.* 2010). In the presence of pro-inflammatory cytokines such as IFN- γ and TNF- α , IECs acquire overexpression of MHC molecules and may function as APCs and activate T cells (Cruickshank *et al.* 2004). Co-culture of IECs from IBD patients has been shown to induce control CD4⁺ T cells to proliferate and produce IFN- γ (Dotan *et al.* 2007). Aberrant TLR signalling in IECs may contribute to the development of intestinal inflammation by altering the response to the intestinal microbiota, thereby facilitating tissue injury and pro-inflammatory responses (Cario 2010). It has been observed that, in the *IL-2*^{-/-} mouse model of colitis, colonic IECs show decreased TLR4 responsiveness and increased TLR2 responsiveness, with up-regulated production of IL-6 and IL-18 (Singh *et al.* 2005). PRR expression by IECs is dysregulated in IBD, with significant up-regulation of TLR4 in IBD, up-regulation of NOD2 in CD, and down-regulation of TLR3 in CD as compared to control subjects (Cario *et al.* 2000; Berrebi *et al.* 2003). Patients with UC show goblet cell and mucin depletion. Moreover, it has been shown that Paneth cells from ileal CD patients with *NOD2* mutations express reduced levels of human α -

defensin 5 and human α -defensin 6 (Wehkamp *et al.* 2005). Moreover, differently from PBMCs from control subjects, PBMCs from CD patients fail to restore defensin production by Paneth cells (Courth *et al.* 2015). CD patients who are homozygous for the T300A *ATG16L1* polymorphism have marked abnormalities in the secretory apparatus of Paneth cells (Cadwell *et al.* 2008). Finally, features of unfolded protein response, which arises from endoplasmic reticulum stress in highly secretory cells such as Paneth cells, have been found to be present both in uninfamed and inflamed IBD epithelium, irrespective of genetic risk variants within this pathway (Kaser *et al.* 2011; Tréton *et al.* 2011).

Aberrant mucosal B cell response in IBD

There is evidence of dysregulated humoral immune response in IBD, and also this alteration may reflect aberrant handling of the intestinal microbiota by the mucosal immune system in this condition, as suggested by the frequent presence of antibodies directed against microbial components and ASCA in CD patients. Unlike CD, UC is often being regarded to as an autoimmune disease, and among the evidences quoted to support this concept are the frequent positivity of serum pANCA and anti-goblet cell autoantibodies, and the presence of genetic risk variants in the the *HLA-DRA* gene (Leiper *et al.* 2011). Moreover, the abnormal mucosal immune cell response in UC has been considered to be driven by B cell-promoting Th2 cells (Fuss *et al.* 2008). However, treatment with rituximab, a humanised antibody against the B cell surface marker CD20, has not been effective in a randomised clinical trial in UC (Leiper *et al.* 2011).

The intestinal lamina propria in IBD is infiltrated by cytotoxic, granzyme B-expressing CD19⁺ and IgA⁺ plasma cells, which may contribute to mucosal damage (Cupi *et al.* 2014). Mucosal production of pIgA, and J chain and pIgR expression are significantly decreased in IBD mucosa. Moreover, in this condition there is a substantial shift from IgA₂ to IgA₁ mucosal synthesis, which has important consequences as this latter subclass is less resistant to proteolytic

degradation. Impaired SIgA-mediated immune exclusion may contribute to the formation of autoantibodies and antibodies against microbial antigens commonly observed in IBD (Brandtzaeg *et al.* 2006). On a different line, it has been shown that faecal bacteria from IBD patients which are highly coated in SIgA confer a higher susceptibility to experimental colitis (Palm *et al.* 2014). Moreover, unlike uncoated microbial species, when SIgA-coated bacteria are transplanted into mice with DSS-induced colitis, they induce a substantial exacerbation of intestinal inflammation (Shapiro *et al.* 2015).

Aberrant mucosal T cell response in IBD

Several *in vivo* and *in vitro* evidences support the importance of both effector T cell overactivation and imbalance between effector T cells and Tregs in triggering and sustaining mucosal inflammation in IBD. A large number of mouse models of colitis, including TNBS-, DSS- and oxazolone-induced, T cell transfer, and IL-10-deficient colitis, have been shown to be associated or dependent on an abnormal T cell response (Maynard *et al.* 2009). Furthermore, inflammatory lesions develop in small intestinal foetal explants cultured *ex vivo* with IL-12 and anti-CD3 (Pender *et al.* 1996), and inhibition of T cell activation, for example upon selective blockade of calcium channels on T cell membrane, leads to a reduction in pro-inflammatory cytokine production both *in vitro* and *ex vivo* (Di Sabatino *et al.* 2009a).

Excessive mucosal T cell activation in IBD may be promoted by the presence of an increased proportion of activated DCs in the lamina propria. Conversely, circulating immature DCs are depleted in active IBD, and display an aberrant response to bacterial CpG-DNA, suggesting a dysregulated interaction with the intestinal microbiota (Baumgart *et al.* 2005). Studies performed using KLH immunisation have shown that oral tolerance is defective in patients with IBD, and also in healthy family members, suggesting that this alteration may contribute to disease susceptibility (Kraus *et al.* 2006). A considerable effort has

been dedicated to the characterisation of the different mucosal effector T cell responses involved in CD and in UC.

Th1 and Th2 cells and cytokines in IBD

An abnormal mucosal Th1 immune response, triggered by increased mucosal levels of IL-18 and IL-12, is present in inflamed intestinal areas in CD (Monteleone *et al.* 1997; Monteleone *et al.* 1999; Podolsky 2002). Indeed, macrophage-derived IL-12 is overexpressed preferentially in CD (Monteleone *et al.* 1997). It has been observed that, while IFN- γ and IL-2 production by T-LPLs is undetectable in the majority of UC patients and control subjects, the proportion of IL-2- and IFN- γ -producing T-LPLs ranges between 3 and 18% in CD patients. This was confirmed by Northern blot analysis, which showed the presence of IFN- γ mRNA in resected colonic mucosa of 5 out of 6 CD patients, as opposed to 1 out of 5 UC patients, and none out of 9 control subjects (Breese *et al.* 1993). Accordingly, IFN- γ production by LPMCs measured by flow cytometry was significantly higher in CD patients compared to UC patients and control subjects (Noguchi *et al.* 1995).

A few years later, it was demonstrated by LPMC isolation and culture and subsequent enzyme-linked immunosorbent assay (ELISA) assays that, upon activation via the CD2/CD28 pathway, CD4⁺ T-LPLs from CD patients release significantly higher IFN- γ concentrations (mean > 85000 pg/ml) than CD4⁺ T-LPLs from UC patients (mean < 60000 pg/ml) or control subjects (mean < 60000 pg/ml) (Fuss *et al.* 1996). Interestingly, upon activation via both the CD2/CD28 and the CD3/CD28 pathways, CD4⁺ T-LPLs from both CD and UC patients produced significantly lower concentrations of IL-4 compared with CD4⁺ T-LPLs from control subjects, nevertheless the mean concentration was lower than 150 ng/ml for all the groups studied (Fuss *et al.* 1996). Furthermore, upon activation of both the CD2/CD28 and the CD3/CD28 pathways, CD4⁺ T-LPLs from UC patients produced significantly higher levels of IL-5 than CD4⁺ T-LPLs from CD

patients and control subjects, nevertheless the mean concentration was lower than 300 ng/ml for all the groups studied (Fuss *et al.* 1996). These results were confirmed by subsequent studies from the same research group, showing that anti-CD2/CD28-activated LPMCs from CD patients produce significantly higher concentrations of IFN- γ (mean > 20000 pg/ml) than UC and control LPMCs (mean < 5000 pg/ml), significantly lower concentrations of IL-4 (mean < 20 pg/ml) than control LPMCs (mean > 60 pg/ml), and significantly lower concentrations of IL-5 (mean < 50 pg/ml) than UC LPMCs (mean > 150 pg/ml) (Fuss *et al.* 2004; Heller *et al.* 2005).

It has been reported that anti-CD2/CD28-activated UC LPMCs release significantly higher concentrations of the Th2 cytokine IL-13 (mean > 900 pg/ml) compared to LPMCs from CD patients and control subjects (mean < 300 pg/ml) (Fuss *et al.* 2004; Heller *et al.* 2005). It has also been reported that the main source of IL-13 in UC mucosa is represented by non-invariant NKT cells, with up to 76% LPMCs being positive for IL-13 after anti-CD2/CD28 activation in UC, as opposed to less than 3% in CD patients and control subjects, and up to 24% being NKT cells, as opposed to 0.5% in CD patients and control subjects (Fuss *et al.* 2004). Subsequently, it has been reported that more than 30% LPMCs the colon of UC patients is constituted by non-invariant NKT cells which respond to stimulation with lyso-sulfatide glycolipid-loaded CD1d-tetramer by producing IL-13 and by enhancing their cytotoxic effect on IECs (Fuss *et al.* 2014). Furthermore, IL-13 transcripts have been found to be detectable in 45% UC colonic samples, whereas they were undetectable in all samples from control subjects, and the frequency of UC samples positive for IL-13 mRNA was significantly higher in inflamed mucosa compared to uninfamed intestine (Kawashima *et al.* 2011). Finally, immunohistochemical characterisation of colonic mucosa showed that phosphorylated STAT6 is absent in paediatric CD patients and control subjects, whereas high expression levels are detectable in paediatric UC patients (Rosen *et al.* 2011).

Nevertheless, there have also been different observations about mucosal Th1 and Th2 cytokines in IBD. Both inflamed CD and UC biopsies cultured *ex vivo* release significantly higher amounts of IFN- γ (mean 234 and 202 pg/ml, respectively) compared to control biopsies (mean 23 pg/ml), with no significant difference between CD and UC (Rovedatti *et al.* 2009). Similar observations have been reported by Bernardo *et al.* (Bernardo *et al.* 2012), who have described the presence of a mixed cytokine profile with predominance of IL-6 and absence of IL-13 in supernatants of UC biopsies cultured *ex vivo*. In another study, significantly lower levels of IL-13 were found in the colonic mucosa of UC patients (median 56 pg/mg of tissue) compared to CD patients and control subjects (82 pg/mg of tissue and 83 pg/mg of tissue, respectively) (Vainer *et al.* 2000). Finally, evaluation of cytokine production by biopsies from paediatric patients with IBD cultured *ex vivo* revealed that IL-13 concentration were significantly lower in supernatants of UC biopsies compared to CD and control supernatants (Kadivar *et al.* 2004).

IFN- γ is a potent pro-inflammatory cytokine produced by Th1 cells and by innate lymphoid cells (ILCs) (Neurath 2014). IFN- γ has been shown to play an important role in the pathogenesis of T cell transfer experimental colitis, and the administration of anti-IFN- γ antibodies to mice soon after T cell transfer prevents the onset of colitis (Powrie *et al.* 1994b). IFN- γ can activate a wide spectrum of immune cells, including endothelial cells, lymphocytes and macrophages, and stimulates macrophages to release TNF- α (Boehm *et al.* 1997). Moreover, IFN- γ enhances the expression of MHC on APCs and promotes neutrophil migration by enhancing the expression of chemokines and their receptors (Colgan *et al.* 1993).

IL-13 is a pleiotropic cytokine produced by T cells and NKT cells, with effects on many cell types, including macrophages, epithelial cells, smooth muscle cells and neurons (Wynn 2003). NKT cell-derived IL-13 has been implicated in the pathogenesis of an experimental model of colitis, namely oxazolone-induced colitis, in which disease can be prevented by treating mice with an IL-13 receptor

(IL-13R) α 2-Fc fusion protein (Heller *et al.* 2002). Interestingly, administration of neutralising antibodies against IL-17E, which is a potent inducer of Th2 cell development, before treatment with oxazolone ameliorates experimental colitis (Camelo *et al.* 2012). On the other hand, the observation that IL-13, in the absence of the decoy receptor IL-13R α 2, may suppress the development and function of Th1/Th17 cells in a murine model of colitis, does not support the pro-inflammatory role of IL-13 in the intestine (Wilson *et al.* 2011). IL-13 activates the pro-apoptotic protease caspase-3 in mouse colonic IECs (Kawashima *et al.* 2011). Moreover, IL-13 has a well-established role in enhancing epithelial permeability via the pore pathway by up-regulating claudin 2 expression within the tight junctions (Weber *et al.* 2010).

Despite the contrasting observations regarding IL-13 expression and function in intestinal inflammation, CD has been thought to be characterised by a Th1 immune response with predominant IFN- γ production, while UC has been considered as a Th2-mediated disease, with excessive production of IL-5 and IL-13. In particular, based on data in experimental colitis, on the observations that IL-13 is up-regulated in UC, and on the *in vitro* establishment of plausible mechanisms of IL-13-mediated intestinal injury, neutralisation of this cytokine has been considered to be a promising therapeutic strategy in UC (Mannon *et al.* 2012).

Th17 cells and cytokines in IBD

After the identification of genetic risk variants within the IL-23-Th17 pathway in IBD, the expression of Th17-associated cytokines has been studied in intestinal inflammation. Significantly higher transcript levels of IL-17A have been detected both in CD and UC mucosa in comparison to the normal intestine (Sugihara *et al.* 2010). Moreover, T-LPLs from both CD and UC patients express significantly higher IL-17A transcript levels compared to those from control subjects (Kobayashi *et al.* 2008). It has been observed by immunohistochemistry that,

while IL-17A is undetectable in the lamina propria of control subjects, high levels of IL-17A are present in CD3⁺ and CD68⁺ cells in the lamina propria of IBD patients (Fujino *et al.* 2003). Moreover, inflamed mucosa from both CD and UC patients cultured *ex vivo* produces significantly higher levels of IL-17A (mean 188 and 130 pg/ml, respectively) than control mucosa (mean 27 pg/ml) (Rovedatti *et al.* 2009). Moreover, the lamina propria of both CD and UC (mean 5.6% and 6.7% of CD3⁺ LPMCs, respectively) patients contains significantly more Th17 cells than the lamina propria of control subjects (mean 2.4% of CD3⁺ LPMCs) (Rovedatti *et al.* 2009).

IL17A is a pleiotropic cytokine expressed mainly by T cells and ILCs, which acts on both immune and non-immune cells (Neurath 2014). IL17R is expressed on a wide range of cells, including fibroblasts, epithelial and endothelial cells (Toy *et al.* 2006). It has been shown that IL-17A induces IL-6 and IL-8 production *in vitro* through mitogen-activated protein kinase (MAPK) pathways, thus favouring the recruitment of neutrophils at sites of inflammation, and triggers T cell proliferation and up-regulation of pro-inflammatory molecules, such as inducible nitric oxide synthase and IL-1 β (Awane *et al.* 1999; Laan *et al.* 2001; Kolls *et al.* 2004).

Th17 cells are also an important source of IL-21, an IL-2-related cytokine which is up-regulated in inflamed IBD mucosa (Monteleone *et al.* 2005; Sarra *et al.* 2010). IL-21 enhances Th1 and Th17 immune responses in the intestine, as shown by the demonstration that IL-21-deficient mice are resistant to Th1/Th17 cell-driven models of colitis and by the observation that IL-17A and IFN- γ production by activated LPMCs from IBD patients is inhibited when IL-21 is blocked (Monteleone *et al.* 2005; Fina *et al.* 2008). Finally, Th17 cells also produce IL-22, which regulates epithelial cell proliferation, wound healing processes, and expression of defensins and Reg3 γ (Pickert *et al.* 2009). It has been observed that IL-22-producing cells are significantly reduced in the inflamed mucosa of UC, but not CD, patients compared to control subjects (Leung *et al.* 2014).

The importance of Th17 cells in intestinal inflammation has been demonstrated *in vivo* in T cell transfer colitis. In particular, it has been shown that, while transfer of T cells lacking the capacity to produce individual Th17 cytokines to RAG-deficient mice is sufficient to induce colitis, transfer of T cells from a ROR γ t-deficient animal fails to induce colitis, indicating that at least one member of Th17 cytokine family is required for intestinal inflammation (Leppkes *et al.* 2009). The observation that blocking the p40 subunit, shared by IL-12 and IL-23, improves both murine colitis and active CD (Sandborn *et al.* 2008) has reinforced the hypothesis that not only the IL-12/Th1 axis, but also the IL-23/Th17 axis, may be playing an important pro-inflammatory role in IBD. However, secukinumab, a monoclonal antibody directed against IL-17A, has been ineffective in active CD (Hueber *et al.* 2012). Indeed, neutralisation of IL-17A and IL-17F alone is ineffective or has a detrimental effect on T cell transfer experimental colitis (O'Connor *et al.* 2009), and treatment with IL-22 protects mice from T cell-dependent colitis (Zenewicz *et al.* 2009). On the other hand, IL-21 deficiency significantly improves experimental colitis (Fina *et al.* 2008). These observations underline the complex effects of Th17 immune response in the intestinal mucosa, which can be pro- or anti-inflammatory depending on the model and the context considered, and on the predominant cytokine expression profile.

Defective mucosal regulatory mechanisms in IBD

Several mucosal immune regulatory mechanisms have been studied in IBD, including TGF- β signalling, Tregs, and IL-17E expression and function.

Both TNBS- and oxazolone-induced colitis are characterised by up-regulated expression of TGF- β in inflamed tissue, associated with impaired TGF- β signalling due to elevated levels of inhibitory Smad7, which in turn leads to reduced phosphorylated Smad3 (Boirivant *et al.* 2006). Moreover, Smad7 overexpression in transgenic mice increases the severity of disease in DSS-

induced colitis by inducing a prominent Th1 response (Rizzo *et al.* 2011). TGF- β expression is increased in the inflamed colonic mucosa of IBD patients with active disease compared to control mucosa (Babyatsky *et al.* 1996). However, pre-incubation of IBD LPMCs with TGF- β is unable to prevent TNF- α -induced NF- κ B activation, implying their resistance to TGF- β -mediated immunosuppression (Monteleone *et al.* 2004). Indeed, TGF- β signalling pathway is defective in CD, as indicated by the reduced levels of phosphorylated Smad3 and Smad3-bound Smad4 in inflamed CD intestinal mucosa (Monteleone *et al.* 2001). This is due to the up-regulation of Smad7 in CD mucosa, where Smad7 is overexpressed by both T cells and non-T cells and critically impairs TGF- β signalling (Monteleone *et al.* 2001).

Transfer of CD4⁺ T cells depleted of Tregs into *RAG*^{-/-} mice results in colitis, and Tregs suppress Th1-mediated experimental colitis (Powrie *et al.* 1994a; Fantini *et al.* 2006). The ability of Tregs to suppress intestinal inflammation in *RAG*^{-/-} mice depends on their release of IL-10 and TGF- β (Maloy *et al.* 2003). Tregs are depleted in peripheral blood of patients with active IBD compared to quiescent IBD patients and control subjects (Singh *et al.* 2001; Chamouard *et al.* 2009). Conversely, there is a higher number of Tregs in the intestinal mucosa of IBD patients compared to control subjects, and Tregs isolated from IBD intestinal mucosa display normal ability to suppress the proliferation of effector T cells *in vitro* (Maul *et al.* 2005; Saruta *et al.* 2007; Eastaff-Leung *et al.* 2010). An intact TGF- β signalling, which is impaired in inflamed IBD mucosa because of up-regulation of the inhibitory molecule Smad7, is required for Treg function (Monteleone *et al.* 2001; Fahlén *et al.* 2005). Effector T cells from IBD intestinal mucosa are hyporesponsive to Treg-mediated suppressive action, and this depends on defective TGF- β signaling due to high Smad7 expression, as T cell responsiveness to Tregs is restored by the culture with Smad7 antisense oligonucleotide (Fantini *et al.* 2009).

IL-17E promotes Th2 cell differentiation and inhibits Th1 and Th17 responses (Monteleone *et al.* 2010). It has been showed that IL-17E, which in the human intestine is expressed mainly by subepithelial macrophages, is significantly reduced in the inflamed intestinal mucosa of subjects with IBD compared with control subjects (Caruso *et al.* 2009). Moreover, the addition of IL-17E to LPMCs isolated from inflamed IBD intestinal areas and stimulated with LPS or peptidoglycan significantly down-regulates the production of IL-12 and IL-23 compared to the condition with LPS or peptidoglycan alone. The defect in the production of IL-17E in IBD could therefore be responsible for the upregulation of IL-12 and IL-23, with expansion of the mucosal Th1 and Th17 cells and consequent tissue damage (Caruso *et al.* 2009).

1.4.5 TNF- α and TNF- α neutralisation in IBD

It is well established that TNF- α expression, both at the transcript and protein levels, is markedly up-regulated in IBD inflamed intestinal mucosa compared to control mucosa (Dionne *et al.* 1997). Moreover, stool TNF- α concentration has been shown to be significantly increased in paediatric CD and UC patients compared to control children (Braegger *et al.* 1992). Immunohistochemical staining has revealed that the median density of TNF- α -expressing cells in the lamina propria of CD and UC patients (148 and 145 cells/mm², respectively) is significantly higher compared to control subjects (5.5. cells/mm²) (Murch *et al.* 1993). It has been shown that a high proportion of CD14⁺ and CD4⁺ LPMCs from IBD patients express transmembrane TNF- α and release soluble TNF- α (Kamada *et al.* 2008; Atreya *et al.* 2011).

TNF- α , which signals via NF- κ B activation, is indeed produced by macrophages, DCs and T cells, and is biologically active as a homotrimeric transmembrane protein and as soluble TNF- α after being cleaved by TACE (Scheller *et al.* 2011; Cabal-Hierro *et al.* 2014). TNF- α receptor (TNFR)2 activation on T cells induces experimental colitis *in vivo* (Holtmann *et al.* 2002), and neutralisation of transmembrane, but not soluble, TNF- α suppresses murine experimental colitis (Perrier *et al.* 2013). TNF- α can exert a wide range of pro-inflammatory effects. In particular, TNF- α induces intestinal barrier impairment by enhancing paracellular permeability via MLCK activation (Clayburgh *et al.* 2005), and by promoting IEC and Paneth cell necroptosis via receptor-interacting protein kinase (RIPK)3 activation (Günther *et al.* 2011). TNF- α also promotes the expression of MAdCAM-1 on endothelial cells *in vitro* (Oshima *et al.* 2001), thereby favouring the recruitment of T cells expressing $\alpha_4\beta_7$ integrin to the intestinal mucosa. Moreover, TNF- α may favour tissue damage by stimulating myofibroblasts to produce MMPs (Okuno *et al.* 2002). Finally, TNF- α promotes macrophage activation *in vitro* (Wesemann *et al.* 2003).

The strongest evidence that TNF- α plays a central role in the pathogenesis of IBD is provided by the therapeutic success of TNF- α neutralisation in IBD. In particular, biologic therapies with agents which neutralise TNF- α have revolutionised the treatment of human chronic inflammatory disease, including IBD (Kuek *et al.* 2007). Responsiveness, however, is difficult to predict, and there may be differences in efficacy and mode of action between different agents (Nesbitt *et al.* 2007; Tracey *et al.* 2008; Siegel *et al.* 2009). For example, infliximab and adalimumab are monoclonal IgG₁ antibodies, whereas etanercept is a dimeric p75 TNF receptor-IgG Fc fusion protein (Sandborn *et al.* 2002; Rutgeerts *et al.* 2009). The fact that etanercept is not effective in CD (Sandborn *et al.* 2001), but is effective in rheumatoid arthritis and psoriasis (Weinblatt *et al.* 1999; Leonardi *et al.* 2003), remains unexplained.

1.4.6 Proteases in IBD intestinal mucosa

Excessive protease expression is a common feature of chronic inflammatory disorders, and elevated tissue levels of proteases are present in rheumatoid arthritis, asthma, chronic obstructive pulmonary disease, and IBD (Baugh *et al.* 1999; Vandooren *et al.* 2004; Elkington *et al.* 2006; Shimshoni *et al.* 2015). In particular, during intestinal inflammation, several proteases are expressed at higher than normal levels, and dysregulated proteolysis leads to both structural and functional alterations in the intestinal barrier, which ultimately promote the development of intestinal lesions.

Protease up-regulation in IBD inflamed mucosa

Dysregulation of protease expression and activity in IBD mucosa has been repeatedly and consistently reported in several studies. MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-10, MMP-12, collagenase 3 (MMP-13) and MMP-14 are all up-regulated in IBD inflamed mucosa (Ravi *et al.* 2007; O'Shea *et al.* 2014; O'Sullivan *et al.* 2015). Conversely, epilysin (MMP-28) is down-regulated in the inflamed mucosa of UC patients compared to control subjects (Rath *et al.* 2010).

Protein extracts from both inflamed and ulcerated intestinal mucosa of both CD and UC patients are characterised by an increased expression of MMP-1, MMP-2, MMP-3, MMP-12, collagenase 3 (MMP-13) and MMP-14 compared to normal intestinal mucosa, and all these enzymes can be produced by stromal and immune cells in the lamina propria (Heuschkel *et al.* 2000; von Lampe *et al.* 2000; Ravi *et al.* 2007). *In situ* hybridisation studies have shown high transcript levels of MMP-1, MMP-3 and MMP-12 in the granulation tissue around intestinal ulcerations of IBD patients, and myofibroblasts in these ulcers also express MMP-13 (Saarialho-Kere *et al.* 1996; Vaalamo *et al.* 1998; Di Sabatino *et al.* 2009b). There is also evidence that another important source of MMP-3 in IBD

mucosa is represented by long-lived lamina propria IgG plasma cells (Gordon *et al.* 2008). MMP-9 is highly up-regulated in the inflamed mucosa of IBD patients, and mucosal MMP-9 levels correlate with disease activity in IBD (Baugh *et al.* 1999). MMP-7 is also highly overexpressed in inflamed IBD mucosa, and its levels correlate with disease activity in UC (Matsuno *et al.* 2003; Salmela *et al.* 2004). The epithelium at the edge of intestinal ulcers in IBD is strongly positive for MMP-7 and MMP-10 (Saarialho-Kere *et al.* 1996; Vaalamo *et al.* 1998). In particular, disruption of the basement membrane below the MMP-7-positive IECs has been reported in IBD (Saarialho-Kere *et al.* 1996), and immediately below MMP-10-expressing IECs there is loss of laminin staining, suggesting enzymatic degradation (Vaalamo *et al.* 1998).

Proteases are dysregulated in IBD not only in terms of expression levels, but also in terms of main producing cell types. For example, in mucosal ulcers of IBD patients, MMP-1 is expressed by macrophages and myofibroblasts, but not by IECs (Arihiro *et al.* 2001). MMP-2 is highly up-regulated in IBD ulcers and is released in excess by IECs, lamina propria myofibroblasts, macrophages and lymphocytes (Kirkegaard *et al.* 2004). MMP-8 and MMP-9, which are essentially undetectable in the normal intestine, are produced in large amounts by IECs during active IBD (Pirilä *et al.* 2003; Ravi *et al.* 2007).

The family of adamalysins is also involved in intestinal inflammation. ADAM15, a transmembrane metalloproteinase with chemotactic effects through its interaction with integrins on the surface of leukocytes, is highly overexpressed by IECs and endothelial cells in inflamed IBD intestine, and it is in close contact with cells expressing its integrin ligand $\alpha_5\beta_1$, hence probably playing a role in inflammatory cell trafficking in this condition (Mosnier *et al.* 2006). Furthermore, the activity of ADAM17 has been reported to be up-regulated in UC but not in CD inflamed mucosa (Brynskov *et al.* 2002). Another study showed an increased ADAM17 expression in inflamed CD mucosa and a correlation between ADAM17 levels and the intensity of neutrophil infiltration (Cesaro *et al.* 2009). The administration

of TNBS to rats stimulates ADAM17 expression and activity at the onset of colitis (Colón *et al.* 2001). Up-regulated ADAM17 activity may contribute to the excess of soluble TNF- α that plays a crucial role in determining mucosal inflammation in IBD. Mucosal expression of TIMP-3, the endogenous inhibitor of ADAM17, is significantly down-regulated in CD patients compared to control subjects, while at the same time ADAM17 activity is up-regulated, resulting in increased shedding of TNF- α from the cell membrane. Interestingly, TIMP-3 is a TGF- β -dependent gene, therefore its reduction in CD may be explained by the impairment in TGF- β signaling present in this condition (Monteleone *et al.* 2012). While ADAM9 and ADAM10 mucosal expression is not different between IBD patients and control subjects, ADAM19 is significantly overexpressed in intestinal IBD biopsies compared to control biopsies (Franzè *et al.* 2013).

Finally, elastinolytic activity is increased in mucosal samples from patients with IBD, and elafin is reduced in the intestinal epithelium (Motta *et al.* 2012). However, it has been reported that transcripts of the natural elastase inhibitor elafin are significantly up-regulated in IBD mucosa compared to control mucosa (Schmid *et al.* 2007), and that also serpin B1, another elastase inhibitor, is increased in the epithelium of IBD patients (Uchiyama *et al.* 2012).

Functional consequences of protease dysregulation in intestinal inflammation

Protease dysregulation contributes to the disruption of intestinal barrier and to the induction of intestinal lesions in IBD by multiple mechanisms (Fig. 1.6), which have been studied using several *in vivo* and *in vitro* experimental models.

Figure 1.6 (see next page). **Simplified representation of MMP-induced intestinal barrier damage in IBD.** IECs, macrophages, LPMCs and myofibroblasts produce large and unbalanced amounts of MMPs in IBD inflamed intestinal mucosa. Once activated by proteolytic cleavage of their pro-domain, MMPs digest the ECM, causing tissue destruction and the development of ulcerations. The alteration of the epithelial barrier allows uncontrolled passage of luminal bacteria, amplifying their interactions with mucosal immune cells and

perpetuating the on-going pro-inflammatory immune response. (Adapted from Biancheri *et al.* 2013).

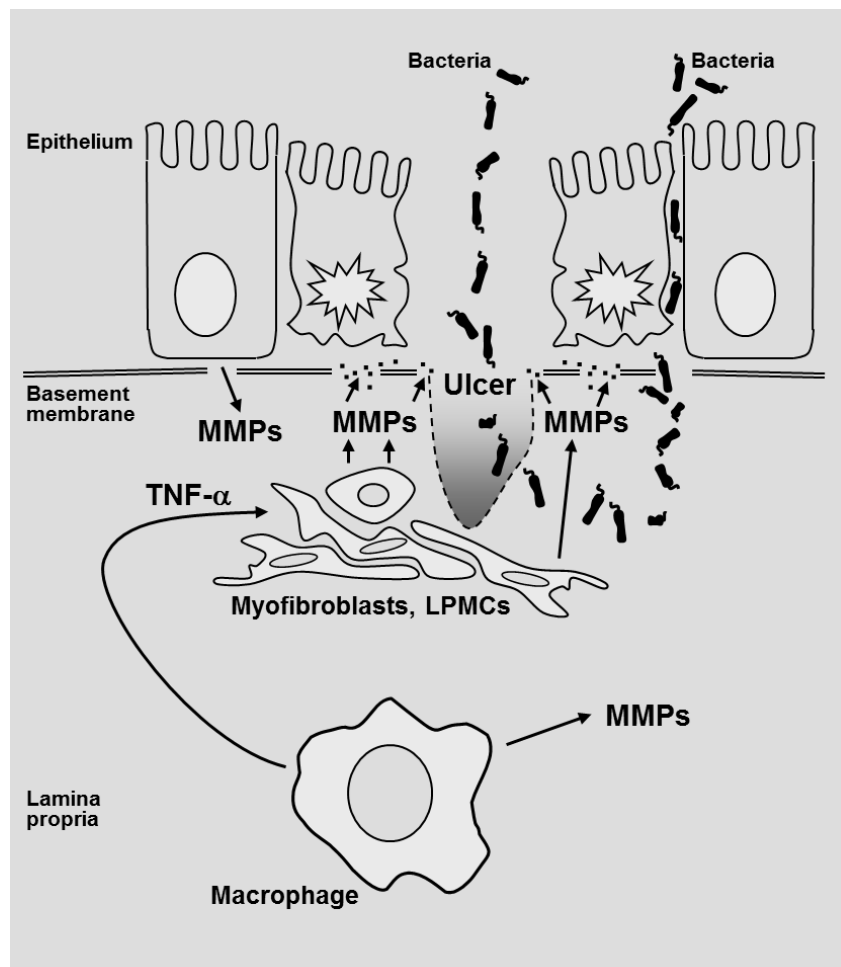


Figure 1.6

The study of mice transgenic for MMPs has provided useful insights into the *in vivo* effect of MMPs in intestinal inflammation. Mucosal MMP-3 and MMP-10 up-regulation precedes the onset of DSS-induced colitis, and RNA silencing of MMP-3 and MMP-10 reduces the severity of DSS-induced colitis (Kobayashi *et al.* 2006). Overexpression of MMP-9 results in reduced MUC2 expression and enhances susceptibility to DSS-induced colitis (Liu *et al.* 2013), whereas MMP-9 deficiency protects mice from DSS- and *S. typhimurium*-induced colitis (Castaneda *et al.* 2005). Interestingly, MMP-9 is up-regulated and localises on the surface of colonic IECs in *C. rodentium*-induced colitis, however MMP-9

deficient mice are equally susceptible to colitis as wild-type mice in this particular model (Rodrigues *et al.* 2012). Fewer evidences are present on the functional role of MMP-12 in intestinal inflammation, however it has been observed that MMP-12-deficient mice are protected against TNBS-induced colitis (Pender *et al.* 2006). Finally, *MMP13*-null mice are protected from DSS-induced colitis, and are resistant to LPS-induced shock. This appears to be due to the ADAM17-like ability of MMP-13 to cleave transmembrane TNF- α into soluble TNF- α , which damages the intestinal barrier (Vandenbroucke *et al.* 2013). Conversely, MMP-2- and MMP-10-deficient mice are more susceptible than wild-type mice to DSS-induced colitis (Garg *et al.* 2006; Koller *et al.* 2012). Similar observations have been made in MMP-7-deficient mice, which are also more susceptible to DSS-induced colitis, possibly due to defective wound healing or enhanced neutrophil migration (Shi *et al.* 2007; Swee *et al.* 2008).

Neutrophil elastase-deficient mice are protected from DSS-induced colitis, treatment with neutrophil elastase inhibitors prevents DSS-induced colitis, and a similar effect is observed upon overexpression of elafin (Morohoshi *et al.* 2006; Motta *et al.* 2011). Likewise, feeding mice lactic acid bacteria engineered to overexpress elafin protects animals from both T cell transfer- and DSS-induced colitis (Motta *et al.* 2012). Neutrophil elastase plays an important role in leukocyte transmigration, and it has been shown that neutrophil elastase-deficient mice have defects in leukocyte adhesion and transmigration (Young *et al.* 2004).

Several *in vitro* observations suggest that dysregulated protease expression has harmful effects on the integrity of the intestinal barrier and mucosa. Culture of human foetal intestinal explants with nanomolar concentrations of recombinant human (rh)MMP-3 causes tissue destruction in 24 hours, and can be prevented by the addition of an MMP-3 inhibitor to the culture (Pender *et al.* 1997). There is evidence that MMP-9 impairs re-epithelialisation, increases endothelial permeability, exerts a disruptive effect on the basement membrane and intercellular junction proteins, activates pro-inflammatory cytokines including IL-

1 β and IL-8 (Mohan *et al.* 2002; Van Den Steen *et al.* 2003; Ravi *et al.* 2007). Furthermore, the addition of MMP-2 and MMP-9 to Caco-2 cells worsens the damage induced by hypoxia *in vitro* (Zitta *et al.* 2012). MMP-12 effects have been studied mainly in atherosclerosis and chronic obstructive pulmonary disease, nevertheless it has been observed that *ex vivo* culture of IBD intestinal biopsies with rhMMP-12 induces epithelial and basement membrane destruction (Pender *et al.* 2006). The action of MMP-7 is complex, as on one hand it has been shown that MMP-7 ensures wound re-epithelisation and activates α -defensins (Wilson *et al.* 1999; Parks *et al.* 2004), whereas on the other hand MMP-7 can also disrupt the intestinal epithelium by degrading the epithelial adherens junction protein E-cadherin (Noë *et al.* 2001). PGP, which is generated by the combined action of MMP-8, MMP-9 and prolyl-endopeptidase, has chemoattractive effects on neutrophils and is up-regulated in IBD mucosa, and this suggests an additional mechanism by which MMPs can sustain the inflammatory infiltrate in this condition (Koelink *et al.* 2014).

Pro-inflammatory cytokines involved in IBD pathogenesis have an important influence on MMP expression. In particular, TNF- α promotes excessive release of a wide range of tissue-degrading MMPs by mucosal myofibroblasts cultured *in vitro* (Okuno *et al.* 2002). Moreover, both IL-21 and IL-17A induce a marked increase in MMP production by intestinal myofibroblasts in synergy with either TNF- α or IL-1 β (Bamba *et al.* 2003; Monteleone *et al.* 2006). Finally, TNF- α , IL-21 and IL-6 stimulate colonic IEC lines and normal colonic explants to overexpress ADAM19, which co-localises with zonula occludens-1, a tight junction-associated protein (Franzè *et al.* 2013).

MMPs exert specific effects on IgG₁. Interestingly, IgG₁ has a Thr-His human neutrophil elastase cleavage site in the upper hinge, and there is a Pro-Glu scissile bond susceptible to cleavage by MMP-3 and MMP-12 in the lower hinge (Brezski *et al.* 2009; Brezski *et al.* 2010). Trastuzumab, a monoclonal antibody used in the treatment of breast cancer, can be degraded by several proteolytic

enzymes with consequent reduction of its immune effector functions (Fan *et al.* 2012). Moreover, cleaved IgG have been detected within squamous cell carcinoma with a predominant localisation at the invasive front, a site particularly rich in proteases (Kinder *et al.* 2013).

1.4.7 Mechanisms of intestinal fibrogenesis in CD

Fibrosis is a common end-stage of chronic inflammatory diseases in various tissues, and involves excessive accumulation of ECM components, including collagen and fibronectin, in the damaged tissue, leading to scarring and organ malfunction (Wynn *et al.* 2012). In CD, where inflammation is transmural, subsequent damage and repair processes may ultimately cause architectural distortion and thickening of all layers of the intestinal wall, thus leading to intestinal fibrosis and stricture development (Burke *et al.* 2007). This represents a significant clinical problem in CD, and patients with fibrostenosing disease may require surgical removal of the affected tract (Rieder *et al.* 2011).

Chronic TNBS-induced murine colitis, which is accompanied by intestinal fibrosis, is characterised by the development of TGF- β -dependent intestinal fibrosis, as shown by the observation that chronic intrarectal TNBS-induced colonic fibrosis, marked by increased mucosal transcripts of TGF- β 1 (Lawrance *et al.* 2003), is effectively prevented by a TGF- β 1 peptide-based vaccine, able to suppress excessive TGF- β 1 activity (Ma *et al.* 2010).

Collagen levels are increased in CD intestinal strictures compared to non-strictured CD and control intestine (Graham *et al.* 1988). In the mucosa overlying CD strictures, MMP-3 and MMP-12 are down-regulated, whereas TIMP-1 is up-regulated, and this may also contribute to the abnormal ECM accumulation observed in CD intestinal fibrosis (Di Sabatino *et al.* 2009b). In a murine model of intestinal fibrosis, TIMP-1, which is up-regulated, effectively inhibits ECM degradation by MMPs (Lawrance *et al.* 2003), and TIMP-1 has been found to be increased in collagenous colitis (Gunther *et al.* 1999). Moreover, in the uninflamed mucosa overlying intestinal strictures of CD patients, expression of the potent pro-fibrotic cytokine TGF- β is higher compared to uninflamed mucosa overlying non-strictured areas (Di Sabatino *et al.* 2009b).

Intestinal myofibroblasts in CD fibrosis

Intestinal myofibroblasts are primarily involved in modulating tissue remodelling through the production of proteases, and play an important role in fibrogenesis and stricture development in CD (Regan *et al.* 2000; Andoh *et al.* 2007; Rieder *et al.* 2009). Myofibroblasts isolated from the mucosa overlying intestinal strictures of CD patients show significantly higher collagen and TIMP-1 production (Fig. 1.7), and have reduced migratory ability compared to those isolated from non-strictured areas of CD patients or from control subjects, suggesting that this cell population exerts a pro-fibrogenic action in CD. Moreover, myofibroblasts isolated from the mucosa overlying CD strictures express increased TGF- β transcripts, and release higher TGF- β protein levels compared to myofibroblasts from uninfamed non-strictured CD and control areas (Di Sabatino *et al.* 2009b).

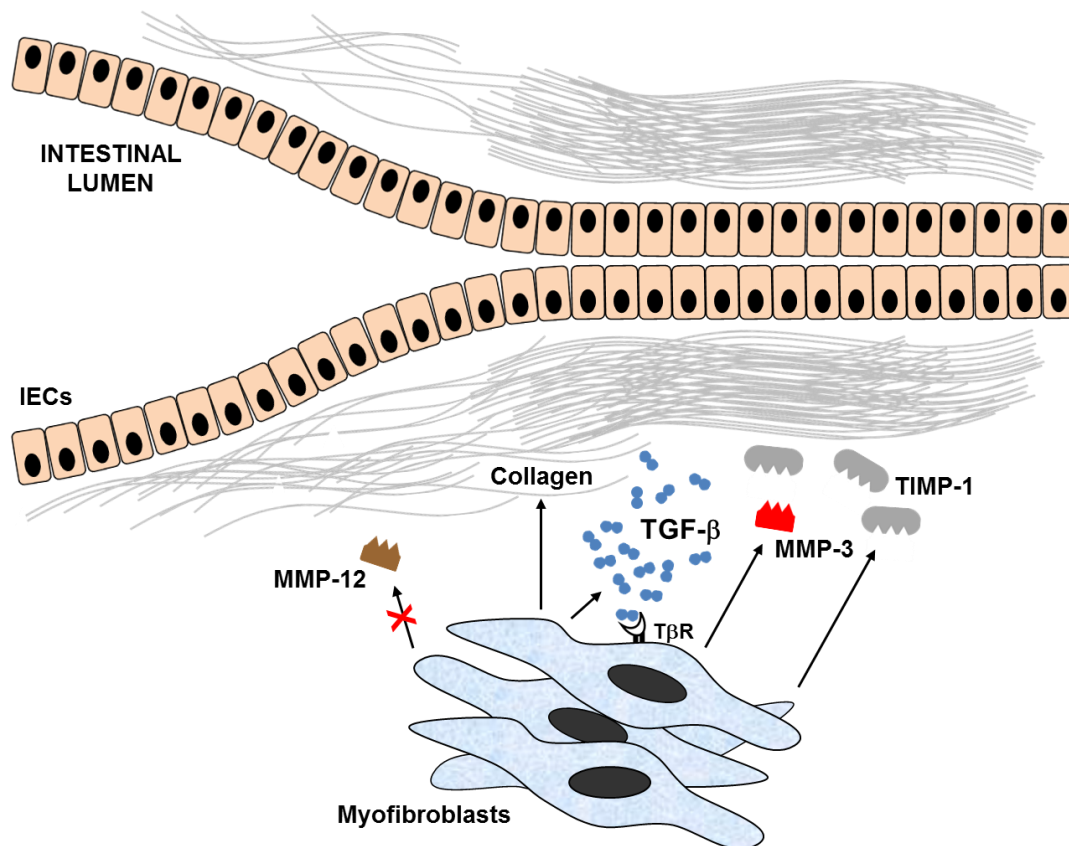


Figure 1.7

Figure 1.7 (see previous page). **TGF- β in CD intestinal fibrosis.** The right part of the diagram represents an intestinal CD stricture, preceded by a pre-stenotic dilatation of an uninfamed non-strictured tract (left part of the diagram). In CD intestinal strictures, myofibroblasts produce excessive amounts of TGF- β . Stricture development in CD is characterised by excessive synthesis and accumulation of collagen and other ECM components by intestinal myofibroblasts, by the reduction of tissue-degrading proteolytic enzymes, such as MMP-3 and MMP-12, and by the increase in TIMPs, including TIMP-1. TGF- β enhances TIMP-1 secretion and reduces MMP-12 production by intestinal myofibroblasts. (Adapted from Biancheri *et al.* 2014).

Within the normal intestinal mucosa, TGF- β plays a pivotal role in the maintenance of immune homeostasis by preventing abnormal and harmful pro-inflammatory responses against the normal constituents of the intestinal flora (Konkel *et al.* 2011), and it is centrally implicated in the physiologic processes of intestinal remodelling and wound healing (Iizuka *et al.* 2011). In fibrostenosing CD, however, TGF- β plays a prominent pro-fibrogenic role by increasing TIMP-1 production by myofibroblasts isolated from strictured, uninfamed non-strictured CD and control mucosa, and by reducing MMP-12 release by myofibroblasts isolated from uninfamed mucosa overlying non-strictured CD and control areas (McKaig *et al.* 2003; Di Sabatino *et al.* 2009b).

In addition to TGF- β , a number of cytokines can modulate myofibroblast function, including IL-1 β and TNF- α (Okuno *et al.* 2002), and those belonging to the IL-17 family (Wynn 2004; Gaffen 2011).

Involvement of IL-17A and IL-17E in tissue fibrosis

IL-17A, which is up-regulated in the intestinal mucosa of patients affected by IBD, has been shown in experimental studies to play an important role in tissue remodelling and fibrosis in a number of different tissues. In particular, IL-1 β -induced and bleomycin-induced lung fibrosis seems to depend on the action of IL-17A, and *IL-17A^{-/-}* mice show reduced susceptibility to experimental skin fibrosis (Okamoto *et al.* 2012; Wilson *et al.* 2010). IL-17A stimulates proliferation and migration of cardiac fibroblasts (Valente *et al.* 2012), promotes hepatic stellate cell activation into fibrogenic myofibroblasts (Meng *et al.* 2012; Tan *et al.* 2013), induces collagen production by skin fibroblasts (Okamoto *et al.* 2012), and promotes epithelial-mesenchymal transition of lung epithelial cells (Mi *et al.* 2011). Unlike acute experimental colitis, chronic TNBS-induced murine colitis is driven predominantly by IL-17A-producing Th17 cells (Guan *et al.* 2011). The observation that targeting IL-23 (a key cytokine in Th17 cell development) with a p40 peptide-based vaccine ameliorates chronic TNBS-induced colitis, and reduces IL-17A, TGF- β levels, and collagen deposition in the intestine (Guan *et al.* 2011), highlights the importance of IL-17A in intestinal experimental fibrosis.

IL-17E exerts two distinct immunological functions: it promotes Th2 response in allergic diseases including asthma (Sharkhuu *et al.* 2006), and it dampens the inflammatory process in immune-mediated disorders, including IBD (Monteleone *et al.* 2010). It has been shown that IL-17E exerts a pro-fibrogenic role in experimental fibrotic disorders. Moreover, IL-17E up-regulates pro-inflammatory cytokine expression and collagen production by lung fibroblasts (Létuvé *et al.* 2006; Gregory *et al.* 2013). IL-17E mediates pulmonary collagen deposition in mice exposed to house dust mite (Gregory *et al.* 2013), and intestinal TNBS-induced fibrosis has been found to be associated in the early phase by a marked increase in IL-17E (Fichtner-Feigl *et al.* 2008a).

Involvement of IL-13 in lung and intestinal fibrosis

IL-13 has been implicated in experimental lung and intestinal fibrosis. Treating mice with bleomycin induces IL-13-dependent pulmonary fibrosis (Fichtner-Feigl *et al.* 2006), and inhibition of IL-13 activity ameliorates TNBS-induced chronic colitis-associated fibrosis (Fichtner-Feigl *et al.* 2008b). IL-13 signalling through IL-13R α 2 induces monocytes and macrophages to release TGF- β (Lee *et al.* 2001; Fichtner-Feigl *et al.* 2006; Fichtner-Feigl *et al.* 2008b), which in turn promotes collagen production (Feagins 2010). Interestingly, Akiho *et al.* (Akiho *et al.* 2005) showed that IL-13 enhances the contractility of smooth muscle cells isolated from the intestine of CD patients, and the study by Bailey *et al.* (Bailey *et al.* 2012) reported higher IL-13 transcripts in the intestinal muscle layer of fibrotic CD.

1.5 Hypotheses

The roles of IL-17, IL-13, MMPs, and ADAM17 are not completely understood in IBD. I shall therefore test the following hypotheses in this Thesis.

IL-17

IL-17A and IL-17E expression in human intestinal fibrosis is not clear. Moreover, no information is available on IL-17R expression in the intestinal mucosa, or on the effect of IL-17A and IL-17E on the release of pro-fibrogenic mediators by intestinal myofibroblasts *in vitro*.

In Chapter 3 of the present Thesis, we therefore tested the following hypotheses:

- “IL-17A and IL-17E expression is dysregulated in fibrostenosing CD intestine”.
- “IL-17A and IL-17E may contribute to intestinal fibrosis in CD”.

IL-13

IL-13 has been reported to be up-regulated in UC intestinal mucosa, and to play an important role in oxazolone-induced colitis and in intestinal experimental fibrosis. However, contrasting observations exist on the levels and the role of IL-13 in inflamed IBD mucosa, and limited information is available on the role of IL-13 in CD intestinal fibrosis.

In Chapter 4 of the present Thesis, we therefore tested the following hypotheses:

- “IL-13 expression is up-regulated in UC intestinal mucosa, and may play a functional role in the mucosal pro-inflammatory response in UC”.
- “IL-13 is up-regulated in CD intestinal strictures, and may contribute to intestinal fibrosis in this condition”.

MMP-3, MMP-9 and MMP-12

Information on the bioavailability of anti-TNF- α agents in inflamed tissues, where they are expected to exert their effect, is lacking. MMPs are up-regulated in IBD inflamed mucosa, and can cleave human IgG₁ near the hinge region. Anti-TNF- α agents in use in clinical practice are IgG₁ or have part of the amino acid sequence in common with Ig.

In Chapter 5 of the present Thesis, we therefore tested the following hypotheses:

- “MMPs and protein extracts from inflamed IBD mucosa impair the integrity and function of anti-TNF- α agents”.
- “Serum levels of cleaved anti-TNF- α agents correlate with primary non-responsiveness to biologic therapy in IBD”.

ADAM17

We performed genetic studies in a family of consanguineous parents and their three children, two of whom had the same clinical features, with skin and small intestine inflammatory lesions, and we identified a deletion in *ADAM17*.

In Chapter 6 of the present Thesis, we therefore tested the following hypotheses:

- “ADAM17 expression is reduced in the skin and small intestine of the affected siblings compared to the unaffected parents and control subjects”.
- “Soluble TNF- α release by PBMCs of the affected siblings is reduced compared to the unaffected parents and control subjects”.

Chapter 2: Materials and Methods

Note: Materials and Methods common to all or most experimental Chapters have been pulled together into this Chapter. Each experimental Chapter is preceded by a Materials and Methods section that is specific for that Chapter alone.

2.1 Patients and tissues

In order to study the role of IL-17 in CD intestinal fibrosis, surgical specimens from ileum or colon of CD patients and control subjects were collected. The role of IL-13 in IBD was studied upon collecting biopsies or surgical specimens from the ileum or colon of IBD patients and control subjects. The effect of proteolytic cleavage on the integrity and the function of anti-TNF- α agents was investigated using colonic biopsies and sera of IBD patients and control subjects. Finally, the disease and immunologic features associated to the deletion in *ADAM17* in two affected siblings of consanguineous parents were studied on skin and duodenal biopsies collected from the family members and control subjects, and on PBMCs from the family members and control subjects. Detailed information on patients, samples and Ethics is reported in the Chapter-specific Patients and tissues paragraph of each experimental Chapter.

2.2 Organ culture

In order to evaluate the *ex vivo* production of IL-17A, IL-17E, IL-6, TNF- α , IL-13, IL-1 β and collagen, and the levels of TGF- β 1 transcripts, intestinal tissue explants (1 mm³ in size) from uninflamed areas of strictured and non-strictured ileum of patients with fibrostenosing CD and from normal ileum of control subjects were placed in 12-well tissue culture plates (BD Biosciences, Oxford, UK; one explant per well) and cultured at 37°C and 5% CO₂ in 800 μ l serum-free HL-1 medium (Cambrex Bioscience, Wokingham, UK) supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin. After 24 hour *ex vivo* culture,

supernatants were collected and stored at -70°C until used for cytokine measurement by ELISA or for collagen measurement by Sircol collagen assay, and intestinal explants were homogenised for subsequent evaluation of TGF- β 1 expression by quantitative reverse transcription (qRT)-polymerase chain reaction (PCR).

In order to evaluate the *ex vivo* production of IL-13, IL-17A, IFN- γ , IL-4 and IL-5 by biopsies from inflamed and uninflamed colonic mucosa of CD and UC patients and control subjects, perendoscopic mucosal biopsies were placed in 24-well tissue culture plates (VWR International, Lutterworth, UK; one biopsy per well) in 300 μ l serum-free HL-1 medium (Cambrex BioScience) supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin, and cultured at 37°C and 5% CO₂. After 24 hour *ex vivo* culture, supernatants were collected and stored at -70°C until used for cytokine measurement by ELISA.

2.3 Myofibroblast isolation and culture

Intestinal submucosal myofibroblasts were isolated as follows. The mucosa, muscle and serosa were dissected away from full thickness samples of bowel and discarded. The remaining tissue was cut into 1 mm-sized samples and cultured at 37°C in a humidified CO₂ incubator in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Poole, UK) supplemented with 20% foetal bovine serum (FBS), 1% non-essential amino acids (Invitrogen Ltd, Paisley, UK), 100 U/ml penicillin, 100 μ g/ml streptomycin, 50 μ g/ml gentamycin, and 1 μ g/ml amphotericin (Sigma-Aldrich). Myofibroblasts migrated out of the tissue after a few days. Established colonies of myofibroblasts were seeded into 25-cm² culture flasks and cultured in DMEM supplemented with 20% FBS and antibiotics. At confluence, the cells were passaged using trypsin-ethylenediaminetetraacetic acid (EDTA) in a 1:2 to 1:3 split ratio. Cells were grown to at least passage 4 before there were enough to use in stimulation experiments.

In order to assess the effect of IL-17A and IL-17E on the production of MMP-3, MMP-12, TIMP-1 and collagen, and the effect of IL-13 on the production of collagen, subconfluent monolayers of myofibroblasts seeded in 12-well plates (BD Biosciences; 3×10^5 cells/well) were starved in serum-free medium for 24 hours at 37°C and 5% CO₂ before being cultured for 24 hours at 37°C and 5% CO₂ with DMEM containing antibiotics in the absence or presence of 10 ng/ml of rhIL-17A, or 10 ng/ml rhIL-17E, or 10 ng/ml rhTNF- α (all from R&D Systems, Abingdon, UK), or 20 ng/ml rhIL-13 (PeproTech EC), or 20 ng/ml rhIL-13 *plus* 20 ng/ml rhTNF- α .

2.4 ELISA

Concentrations of IL-17A and IL-17E in tissue sample homogenates were measured using specific ELISA kits (all from R&D Systems), in accordance with the manufacturer's instructions, and were normalized to the total protein concentration, determined by a protein assay (Bio-Rad Laboratories, Hemel Hempstead, UK). Concentrations of IL-17A, IL-17E, IL-6, TNF- α , IL-13, IFN- γ , IL-4, IL-5 and IL-1 β in culture supernatants and tissue homogenates were assessed using specific ELISA kits (all from R&D Systems), in accordance with the manufacturer's instructions. With regards to the sensitivity of the ELISA kits, the detection range of each kit is as follows: IL-17A, 15.6-1000 pg/ml; IL-17E, 46.9-3000 pg/ml; IL-6, 9.4-600 pg/ml; TNF- α , 15.6-1000 pg/ml; IL-13, 15.6-1000 pg/ml; IFN- γ , 15.6-1000 pg/ml; IL-4, 31.2-2000 pg/ml; IL-5, 23.4-1500 pg/ml; IL-1 β , 3.9-250 pg/ml. Since IL-13 measurements were central to my study, I also repeated the measurement of IL-13 in culture supernatants using a different ELISA kit (eBioscience, San Diego, CA), with higher sensitivity (detection range 4-500 pg/ml) compared to the correspondent R&D Systems kit.

2.5 Collagen assay

Total soluble forms of collagen were measured in supernatants of tissue explants

and myofibroblasts using the Sircol Collagen Assay Kit (Biocolor Ltd, Belfast, UK), in accordance with the manufacturer's instructions. The collagen content in each sample was calculated as an average of three readings.

2.6 RNA extraction and analysis of mRNA expression by qRT-PCR

In order to assess the expression of IL-13, IL-5, IL-17A and IFN- γ in IBD and control mucosa, and in order to evaluate the expression of TGF- β 1 in CD and control explants, RNA was extracted from snap-frozen mucosa of UC, CD and control subjects, and from cultured tissue explants. cDNAs were synthesized (Improm-II RT system; Promega, Southampton, UK) using random hexamers and 1 μ g of RNA in a final volume of 20 μ l. RT reactions were performed using the Improm-II reverse transcriptase enzyme from the kit. An RT reaction without reverse transcriptase enzyme was performed for each tissue type as a negative control for qPCR. IL-13, IL-5, IL-17A, IFN- γ and TGF- β 1 primers and probe sets were validated for use with the $\Delta\Delta C_T$ method of quantification. The probe was labeled with a 59-reporter dye FAM (6-carboxy-fluorescein) and the 39-quencher dye TAMRA (6-carboxy-N,N,N',N9-tetramethyl-rhodamine). RT reactions were diluted 1 in 10 in distilled water, and 5 μ l of template was added to 6.5 μ l of 2x master mix (Eurogentech, Seraing, Belgium) containing 1.2 μ mol/l forward and reverse primers and 0.248 μ mol/l of probe in a total volume of 12.5 μ l. The PCR protocol was as follows: 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute. Thermocycling and real-time detection of PCR products were performed on a sequence detection system (iCycler iQ; Bio-Rad Laboratories). Expression levels were normalized against 18S, β -actin, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and values were calculated using the $\Delta\Delta C_T$ method.

2.7 Western blotting

In order to assess IL-17A, IL-17E, IL-17RB and IL-17RC expression in CD and control mucosa, in order to determine MMP-3, MMP-12 and TIMP-1 release by IL-17-stimulated CD and control myofibroblasts, in order to evaluate claudin 2 expression by IL-13-stimulated T84 cells, and in order to study ADAM17, ADAM10 and DSG1/2 expression by keratinocytes, tissue samples or myofibroblasts or T84 cell monolayers or keratinocytes were homogenised in ice-cold lysis buffer, and the amount of protein was determined by the DC Protein Assay (Bio-Rad Laboratories). Equal amounts of protein or 15 µl of cell culture supernatants were loaded into each lane and run in 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels under reducing conditions. After electrophoresis, protein was transferred onto nitrocellulose membranes (Bio-Rad Laboratories). Membranes were blocked with 5% non-fat dry milk, followed by incubation for 16 hours at 4°C with the following antibodies: goat anti-human IL-17A (1 µg/ml), goat anti-human IL-17E (0.1 µg/ml) (both from R&D Systems), mouse anti-human IL-17RB (1:500 dilution; LifeSpan Biosciences, Seattle, WA), mouse anti-human IL-17RC (1:750 dilution), rabbit anti-human MMP-3 (1:200 dilution), rabbit anti-human MMP-12 (1:1000 dilution) (all three from Abcam, Cambridge, UK), mouse anti-human TIMP-1 (1 µg/ml; Oncogene Research, Nottingham, UK), rabbit anti-human ADAM17 (1/500; Abcam), rabbit anti-human ADAM10 (1:500; Millipore, Bedford, MA), or mouse anti-human DSG1/2 (1 µg/ml; Zymed, Cambridge, UK). Appropriate antibodies conjugated to horseradish peroxidase (Dako, High Wycombe, UK) were used as secondary antibodies. For the detection of claudin 2 expression by T84 cells, rabbit anti-human claudin 2 antibody (2 µg/ml; Invitrogen Ltd) and a goat anti-rabbit IgG antibody conjugated to horseradish peroxidase (Dako) were used as primary and secondary antibodies, respectively. Reactions were developed with enhanced chemiluminescence (ECL Plus Kit; Amersham Biosciences, Little Chalfont, UK). Blots were then stripped and analysed for β-actin, as an internal loading control, using a rabbit anti-β-actin antibody (1:5000 dilution; Abcam). Blots on T84 cell

lysates were stripped and analysed for cytokeratin 19 as a loading control for epithelial proteins, using a mouse anti-human cytokeratin 19 antibody (1:1000 dilution; Abcam). Bands were quantified by scanning densitometry using an LKB Ultrascan XL Laser Densitometer (Kodak Ltd, Hemel Hempstead, UK).

The effect of proteases on the integrity of TNF- α neutralising agents was assessed by immunoblotting under denaturing conditions. After co-incubation with active rhMMP-3 or rhMMP-12 or rhMMP-9 or IBD mucosal homogenates or control mucosal homogenates, TNF- α neutralising agents were run on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis gels under reducing or non-reducing conditions. After electrophoresis, proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories). Membranes were then incubated with a rabbit anti-human Ig Fc antibody (1:3000 dilution; Fisher Scientific UK, Loughborough, UK), or a rabbit anti-human Ig κ light chain antibody (1:2000 dilution; Abcam) followed by a horseradish peroxidase-conjugated goat anti-rabbit Ig secondary antibody (Dako). Bands were quantified by scanning densitometry using ImageJ software program (NIH, Bethesda, MD).

2.8 PBMC isolation

PBMCs were isolated from heparinised peripheral venous blood within 10 minutes from collection by Ficoll-Hypaque (Sigma-Aldrich) density-gradient centrifugation. Sample was centrifuged (1500 rpm, 30 minutes) and the resultant 'buffy coat' containing the PBMCs collected. After further centrifugation (1500 rpm, 10 minutes) and washing in 10 ml PBS containing EDTA (2 mM), the final cell pellet was resuspended in 10 ml PBS containing EDTA (2 mM). Cells were counted and the final concentration adjusted to 1×10^6 cells/ml by resuspension in RPMI-1640 medium containing 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin, and kept on ice until used. The total processing time from collection to culture was less than 1 hour.

2.9 Statistical analysis

Data were analysed by the GraphPad Prism statistical PC program (GraphPad Software, San Diego, CA) using the paired or unpaired t test, the Mann-Whitney U -test, or using the ANOVA One way analysis of variance followed by the Tukey's multiple comparison test or by the Dunnett's multiple comparison test.

Chapter 3: The role of IL-17A and IL-17E in CD intestinal fibrosis

Note: The wound-healing scratch assay on intestinal myofibroblasts and the analysis of the data (Paragraph 3.3.7) have been performed by Professor Antonio Di Sabatino's group in the Laboratory of the First Department of Internal Medicine, St Matteo Hospital, University of Pavia, Pavia, Italy, and primary data are not available. These results have been published in the following paper, on which I am first Author (Biancheri P, Pender SL, Ammoscato F, Giuffrida P, Sampietro G, Ardizzone S, Ghanbari A, Curciarello R, Pasini A, Monteleone G, Corazza GR, MacDonald TT, Di Sabatino A. The role of interleukin 17 in Crohn's disease-associated intestinal fibrosis. *Fibrogenesis Tissue Repair* 2013;6:13).

IL-17A and IL-17E expression in human intestinal fibrosis is not clear. Moreover, no information is available on IL-17R expression in the intestinal mucosa, or on the effect of IL-17A and IL-17E on the release of pro-fibrogenic mediators by intestinal myofibroblasts *in vitro*. Further information on the background to this Chapter is present in Section "1.4.7 Mechanisms of intestinal fibrogenesis in CD".

3.1 Aims of the study

This study aims to explore the possible involvement of IL-17A and IL-17E in CD intestinal fibrosis using the following approach:

1. Assessment of the expression of IL-17A and IL-17E in the intestine of patients with fibrostenosing CD and in the normal intestine of control subjects.
2. Investigation of IL-17A and IL-17E production by intestinal tissue explants from CD strictured areas cultured *ex vivo*.
3. Assessment of IL-17R (IL-17RC for IL-17A and IL-17RB for IL-17E, respectively) expression in the intestinal tissue and myofibroblasts from patients with fibrostenosing CD and from normal intestine of control subjects.
4. Evaluation of the *in vitro* effects of IL-17A and IL-17E on CD and control intestinal myofibroblasts.

3.2 Chapter-specific Materials and Methods

3.2.1 Patients and tissues

Surgical specimens were collected from uninfamed areas of strictured and non-strictured ileum or colon of 29 patients with fibrostenosing CD (Table 3.1). Diagnosis of CD was ascertained by standard clinical criteria (Van Assche *et al.* 2010), and strictured areas were identified by colonoscopy, entero-magnetic resonance imaging (MRI), or enteroclysis (Maglinte *et al.* 2005). None of the patients with CD had been treated previously with ciclosporin, tacrolimus, methotrexate, or anti-TNF- α antibodies. Intestinal samples were also collected from macroscopically and microscopically unaffected (at least 500 mm from the tumour mass) ileum or colon of 27 patients undergoing intestinal resection for colon cancer (Table 3.2), who were considered as control subjects. CD patients and control subjects were sex-matched, whereas control subjects were significantly ($p < 0.0001$) older than CD patients, most likely due to differences in the age of clinical presentation between CD and colon cancer. This study was approved by the NRES Committee London - City & East (REC reference: 10/H0704/74), and informed consent was obtained from all patients and control subjects.

Table 3.1. Clinical features of patients with fibrostenosing CD (n=29)

Characteristics and parameters	N.	Median (range)
Age (years)		35.8 (23-61)
Sex (M/F)	16/13	
First attack	6	
Intestinal location		
<i>Small bowel and colon</i>	15	
<i>Small bowel only</i>	9	
<i>Colon only</i>	5	
Duration of disease (months)		81.2 (7-196)
Number of recurrences		4 (0-9)
CDAI		223 (155-426)
Treatment		
<i>Mesalazine</i>	13	
<i>Topical steroids</i>	3	
<i>Antibiotics</i>	9	
<i>Azathioprine/6-MP/methotrexate</i>	14	

6-MP, 6-mercaptopurine.

Table 3.2. Clinical features of control subjects (n=27)

Characteristics and parameters	N.	Median (range)
Age (years)		53.4 (45-71)
Sex (M/F)	13/14	
Comorbidities		
<i>Ischaemic heart disease</i>	4	
<i>Hypertension</i>	7	
<i>Hyperlipaemia</i>	6	
<i>Diabetes</i>	6	
<i>Chronic kidney disease</i>	2	
<i>None</i>	14	
Current medications		
<i>Anti-hypertensive</i>	7	
<i>Diuretics</i>	5	
<i>Anti-coagulants</i>	1	
<i>Aspirin</i>	4	
<i>Statins</i>	6	
<i>Metformin</i>	6	
<i>Proton pump inhibitors</i>	8	
<i>Anti-depressants</i>	2	
<i>None</i>	9	

F, female; M, male.

3.2.2 Study design

Since the excessive collagen deposition occurs in the submucosa and outer muscle layers, the mucosa was discarded upon dissection, and the muscle-enriched fraction was used. Tissue from the ileum and colon of 12 CD patients and 11 control subjects was homogenised in order to study *in vivo* IL-17A, IL-17E, IL-17RC and IL-17RB tissue expression (Fig. 3.1, Fig. 3.2). The *ex vivo* tissue production of IL-17A, IL-17E and pro-fibrogenic mediators was evaluated in the supernatants of cultured tissue explants from the ileum of further six CD patients and seven control subjects (Fig. 3.3). Intestinal myofibroblasts were isolated from the ileum and colon of further 11 CD patients and nine control subjects, and used for experiments aimed at determining IL-17RC and IL-17RB expression (Fig. 3.4), MMP, TIMP and collagen production (Fig. 3.5, Fig. 3.6), and migration (Fig. 3.7) upon stimulation with IL-17A or IL-17E.

3.2.3 Wound-healing scratch assay

Myofibroblast migration was assessed in accordance with the modified method of Rodriguez *et al.* (Rodriguez *et al.* 2005; Di Sabatino *et al.* 2009b). Briefly, cells (2×10^5) were seeded into cell culture dishes (Nalge Nunc International, Rochester, NY, USA) with 2 mm grids, size 35 x 10 mm, in 2 ml of DMEM supplemented with 20% FBS and antibiotics. The cells were maintained at 37°C and 5% CO₂ until confluent. Once confluent, each dish of monolayer cells was given a mechanical wound by scoring with a 200 µl pipette tip, parallel to the grid bars along the central grid line. This permitted easy viewing of the cells growing back together, and ensured that the 2 mm grid could be used as a reference, so that the wound areas could be measured and compared. Wound placement was checked with an inverted microscope (CK2; Olympus UK Ltd, London, UK). The medium was then removed, and the cells were washed five times with HL-1 serum-free medium (Cambrex Bioscience) supplemented with antibiotics, and then replaced with 1.5 ml HL-1 medium with or without 10 ng/ml rhIL-17A or rhIL-

17E (both from R&D Systems). Photographs of the cells in each grid along the induced wound were taken at 0, 2, 4, 8, 16 and 24 hours, using a digital camera (Camedia; Olympus UK Ltd) with 34 to 40 zoom, and 20x magnification, attached to a light microscope. The computer program Image J was used to measure the area of initial damage (images taken at time 0) and of the remaining damage at subsequent time points. Each grid image was observed separately, and two points per grid at the same position at every time point were measured using imaging software at the same magnification. The percentage of wound repair was then calculated.

3.3 Results

3.3.1 *In vivo* IL-17A and IL-17E tissue expression

IL-17A and IL-17E levels were analysed by immunoblotting in tissue samples collected from uninfamed areas of strictured and non-strictured ileum and colon of 12 patients with fibrostenosing CD and from normal ileum and colon of 11 control subjects (Fig. 3.1A). IL-17A expression was significantly ($p<0.001$) up-regulated in strictured CD areas compared with non-strictured CD areas and control ileum and colon. No significant difference in IL-17A expression was found between non-strictured CD areas and control ileum and colon. IL-17E levels did not differ significantly between strictured and non-strictured CD areas and control ileum and colon.

In parallel, IL-17A and IL-17E expression was analysed by ELISA in the same samples (Fig. 3.1B). IL-17A was significantly up-regulated in strictured CD (mean 98.5 ± 13.7 pg/ μ g total protein) compared with non-strictured CD (mean 42.3 ± 11.0 pg/ μ g total protein, $p<0.001$) and control (mean 46.5 ± 8.3 pg/ μ g total protein, $p<0.001$) areas. There was no significant difference in IL-17A expression between non-strictured CD and control areas. IL-17E did not significantly differ between strictured CD (mean 222.5 ± 32.4 pg/ μ g total protein), non-strictured CD (mean 178.1 ± 30.3 pg/ μ g total protein), and control areas (mean 188.2 ± 35.3 pg/ μ g total protein).

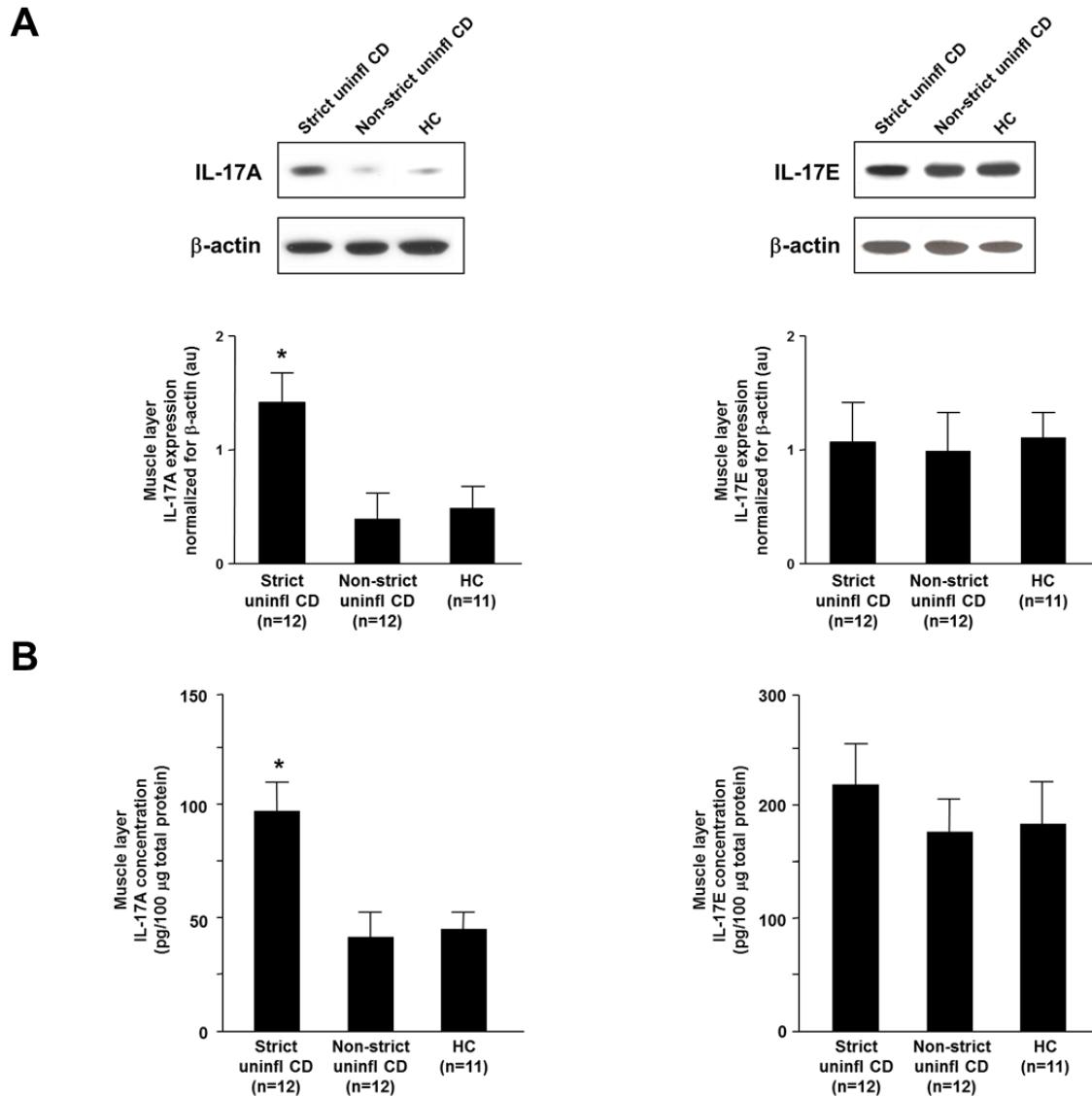


Figure 3.1

Figure 3.1. *In vivo* expression of IL-17A and IL-17E. IL-17A and IL-17E were detected by both (A) immunoblotting and (B) ELISA in uninflamed strictured (Strict uninfl) and non-strictured (Non-strict uninfl) ileum and colon of 12 patients with fibrostenosing CD and from normal ileum and colon of 11 control subjects (HCs). (A) Each blot shown in the upper panel displays IL-17A and IL-17E expression in uninflamed strictured and non-strictured ileum of one patient with fibrostenosing CD and from normal ileum of one HC, and is representative of experiments performed in 12 patients with CD and 11 HCs. Blots were stripped and analysed for β-actin as an internal loading control. In the lower panel, densitometry of IL-17A and IL-17E expression normalised for β-actin is shown. Results are mean ± SEM. au, Arbitrary units. (B) Results, expressed as pg/100 μg of total protein, are mean ± SEM. Data were analysed by ANOVA One way

analysis of variance followed by the Tukey's multiple comparison test. * $p < 0.001$ versus Non-strict uninfl and HC tissue samples.

3.3.2 *In vivo* IL-17RC and IL-17RB tissue expression

IL-17RC and IL-17RB expression was then analysed by immunoblotting in the same tissue samples (Fig. 3.2). IL-17RC expression was not significantly different between CD strictured, CD non-strictured, and control ileum and colon (Fig. 3.2A). Similarly, IL-17RB expression did not differ between CD strictured, CD non-strictured and control ileum and colon (Fig. 3.2B).

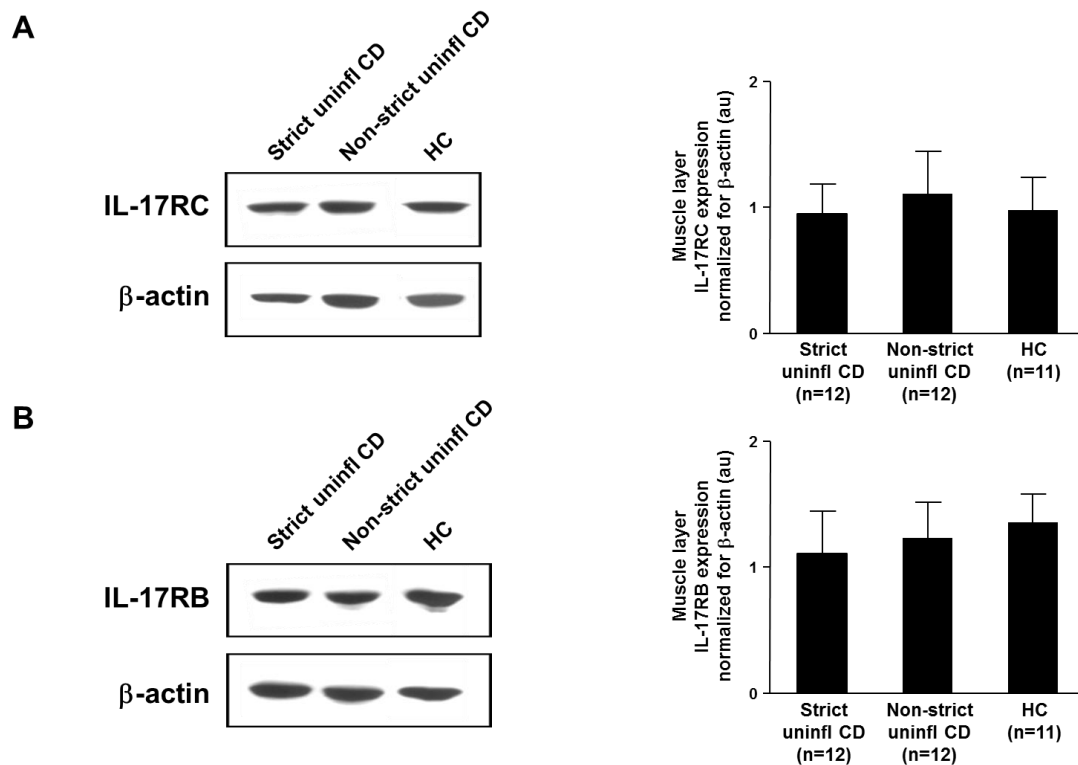


Figure 3.2

Figure 3.2. *In vivo* expression of IL-17RC and IL-17RB. (A) IL-17RC and (B) IL-17RB were detected by immunoblotting in uninflamed areas of strictured (Strict uninfl) and non-strictured (Non-strict uninfl) ileum and colon of 12 patients with fibrostenosing CD and from normal ileum and colon of 11 control subjects (HCs). Each blot shown in the left panels displays IL-17RC and IL-17RB expression in uninflamed strictured and non-strictured ileum of one patient with fibrostenosing CD and from normal ileum of one HC, and is representative of

experiments performed in all patients with CD and all HC subjects. Blots were stripped and analysed for β -actin as an internal loading control. In the right panels, densitometry of IL-17RC and IL-17RB expression normalized for β -actin is shown. Results are mean \pm SEM. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test. au, Arbitrary units.

3.3.3 *Ex vivo* tissue production of IL-17A, IL-17E, IL-6, TNF- α , collagen and TGF- β 1

Tissue explants (1 mm^3 in size) from uninflamed areas of strictured and non-strictured ileum of six patients with fibrostenosing CD and from normal ileum of seven control subjects were cultured *ex vivo* for 24 hours, and IL-17A, IL-17E, IL-6, TNF- α , and collagen concentration in the culture supernatant and TGF- β 1 transcripts in the cultured tissue were measured (Fig. 3.3). IL-17A was significantly higher in the organ culture supernatants of strictured CD (mean $110.0 \pm 23.8\text{ pg/ml}$) than in those of non-strictured CD (mean $55.9 \pm 8.8\text{ pg/ml}$, $p < 0.05$) or normal ileum (mean $51.1 \pm 8.9\text{ pg/ml}$, $p < 0.05$). Concentrations of IL-17E, IL-6, and TNF- α did not differ significantly between the supernatants of strictured CD (mean $653.3 \pm 283.5\text{ pg/ml}$, $26553 \pm 6647\text{ pg/ml}$, and $43.4 \pm 7.6\text{ pg/ml}$, respectively), non-strictured CD (mean $1028.0 \pm 202.5\text{ pg/ml}$, $31740 \pm 3944\text{ pg/ml}$, and $40.8 \pm 7.7\text{ pg/ml}$, respectively), and normal ileum (mean $1068.0 \pm 282.8\text{ pg/ml}$, $36784 \pm 2516\text{ pg/ml}$, and $30.0 \pm 7.9\text{ pg/ml}$, respectively). Collagen concentration was significantly higher in the supernatants of strictured CD (mean $979.0 \pm 108.6\text{ }\mu\text{g/ml}$, respectively) than in those of non-strictured CD (mean $604.6 \pm 97.5\text{ }\mu\text{g/ml}$, $p < 0.05$) and normal ileal areas (mean $523.6 \pm 77.9\text{ }\mu\text{g/ml}$, $p < 0.01$). TGF- β 1 transcripts were significantly higher in the cultured tissue explants from strictured CD than in those from non-strictured CD ($p < 0.01$) and normal ileum ($p < 0.001$).

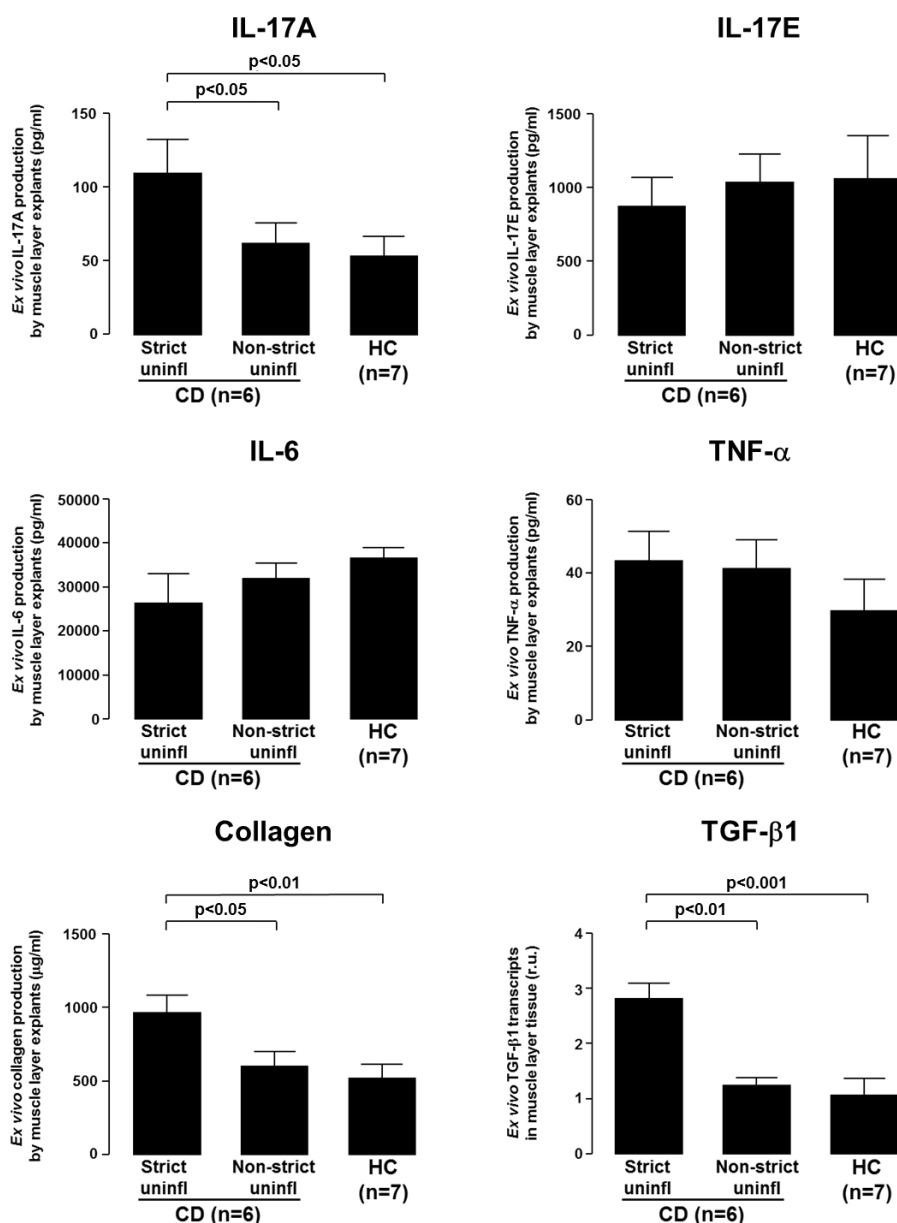


Figure 3.3

Figure 3.3. Levels of cytokines and pro-fibrogenic mediators in tissue explant culture supernatants. Levels of IL-17A, IL-17E, IL-6, and TNF- α , expressed as pg/ml, and collagen, expressed as μ g/ml, in the supernatants of tissue explants from uninflamed areas of strictured (Strict uninfl) and non-strictured (Non-strict uninfl) ileum of six patients with fibrostenosing CD and from normal ileum of seven control subjects (HCs), cultured for 24 hours in the absence of stimuli, and levels of TGF- β 1, expressed as relative units compared with the median expression in HCs (which was assigned the value 1), in the same cultured tissue explants. Values are mean \pm SEM. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test. r.u., Relative units.

3.3.4 IL-17RC and IL-17RB expression on myofibroblasts

IL-17RC and IL-17RB expression was then studied by immunoblotting in lysates of myofibroblasts isolated from uninfamed areas of strictured and non-strictured ileum and colon of six patients with fibrostenosing CD, and from normal ileum and colon of seven control subjects (Fig. 3.4). Myofibroblasts isolated from strictured CD areas, non-strictured CD areas, and control ileum and colon expressed both IL-17RC (Fig. 3.4A) and IL-17RB (Fig. 3.4B). However, no significant difference in IL-17RC and IL-17RB expression was found between strictured, non-strictured CD, and control myofibroblasts.

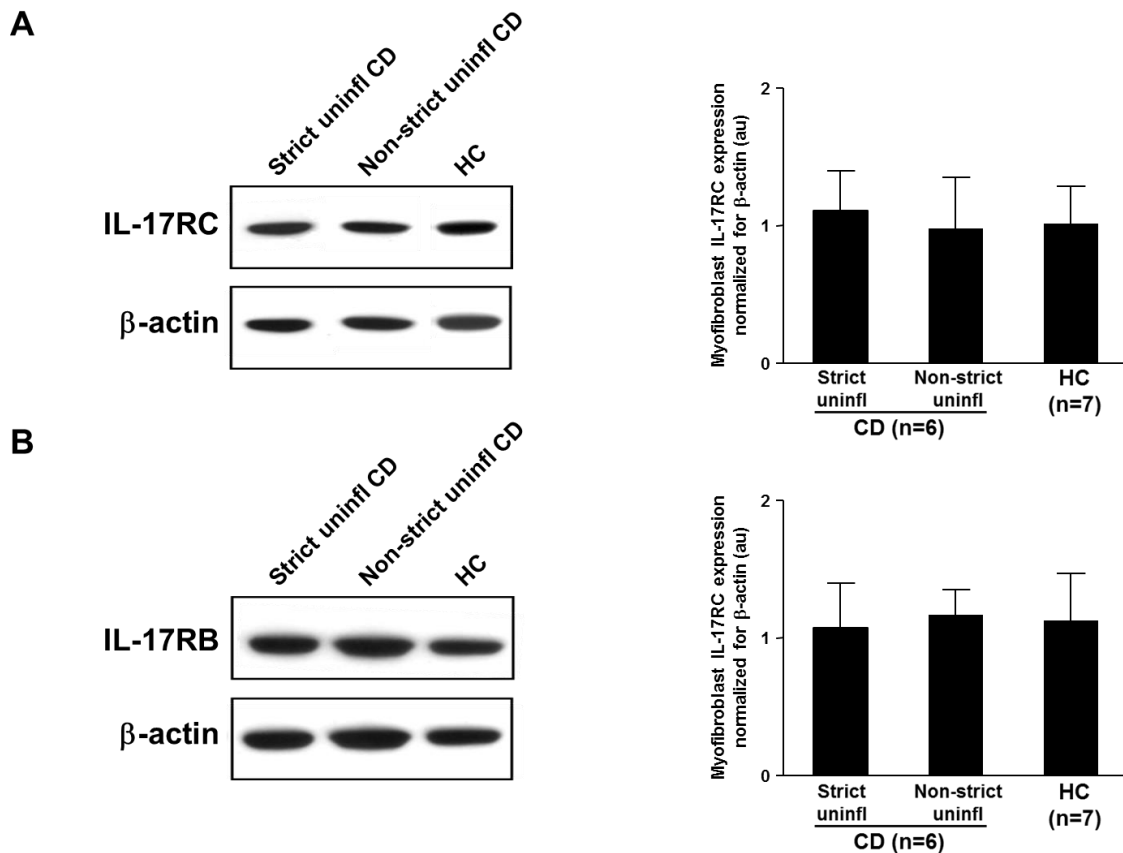


Figure 3.4

Figure 3.4. Expression of IL-17RC and IL-17RB on intestinal myofibroblasts. (A) IL-17RC and (B) IL-17RB were detected by immunoblotting on lysates of myofibroblasts isolated from uninfamed areas of strictured (Strict uninfl) and non-strictured (Non-strict uninfl) ileum and colon of six patients with fibrostenosing CD and from normal ileum and colon of seven control subjects (HCs). Each blot shown in the left panels displays IL-17RC and IL-17RB

expression in myofibroblasts from uninflamed strictured and non-strictured colon of one patient with fibrostenosing CD and from normal colon of one HC, and is representative of experiments performed in all patients with CD and all HC subjects. Blots were stripped and analysed for β -actin as an internal loading control. In the right panels, densitometry of IL-17RC and IL-17RB expression normalized for β -actin is shown. Results are mean \pm SEM. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test. au, Arbitrary units.

3.3.5 *In vitro* effect of rhIL-17A and rhIL-17E on MMP-3, MMP-12, and TIMP-1 production by myofibroblasts

The production of MMP-3, MMP-12, and TIMP-1 was evaluated by immunoblotting in supernatants of myofibroblasts from strictured or non-strictured ileum and colon of six patients with fibrostenosing CD, and from normal ileum and colon of six control subjects, stimulated with rhTNF- α , rhIL-17A, or rhIL-17E (Fig. 3.5). Stimulation with rhIL-17A and rhIL-17E induced a significant ($p < 0.05$) increase in MMP-3 and MMP-12 production by CD myofibroblasts from strictured and non-strictured areas and by control myofibroblasts. Stimulation with rhTNF- α induced a significant ($p < 0.05$) increase in both MMP-3 and MMP-12 production compared to control (myofibroblasts from the same group of patients cultured with medium alone). No significant difference in MMP-3 or MMP-12 production was found between rhTNF- α , rhIL-17A, and rhIL-17E stimulation (Fig. 3.5A,B). Myofibroblasts from CD strictured areas showed a significantly ($p < 0.05$) lower spontaneous release of MMP-12 than myofibroblasts from CD non-strictured areas or control ileum and colon (Fig. 3.5B). Myofibroblast stimulation with rhIL-17A induced a significant ($p < 0.05$) increase in TIMP-1 production compared with unstimulated cells. Stimulation with rhIL-17E did not induce any significant change in TIMP-1 production compared to control (myofibroblasts from the same group of patients cultured with medium alone). Stimulation with rhTNF- α induced a significant ($p < 0.05$) increase in TIMP-1 production compared to control (myofibroblasts from the same group of patients cultured with medium alone). No

significant difference in TIMP-1 production was found between rhTNF- α and rhIL-17A stimulation (Fig. 3.5C).

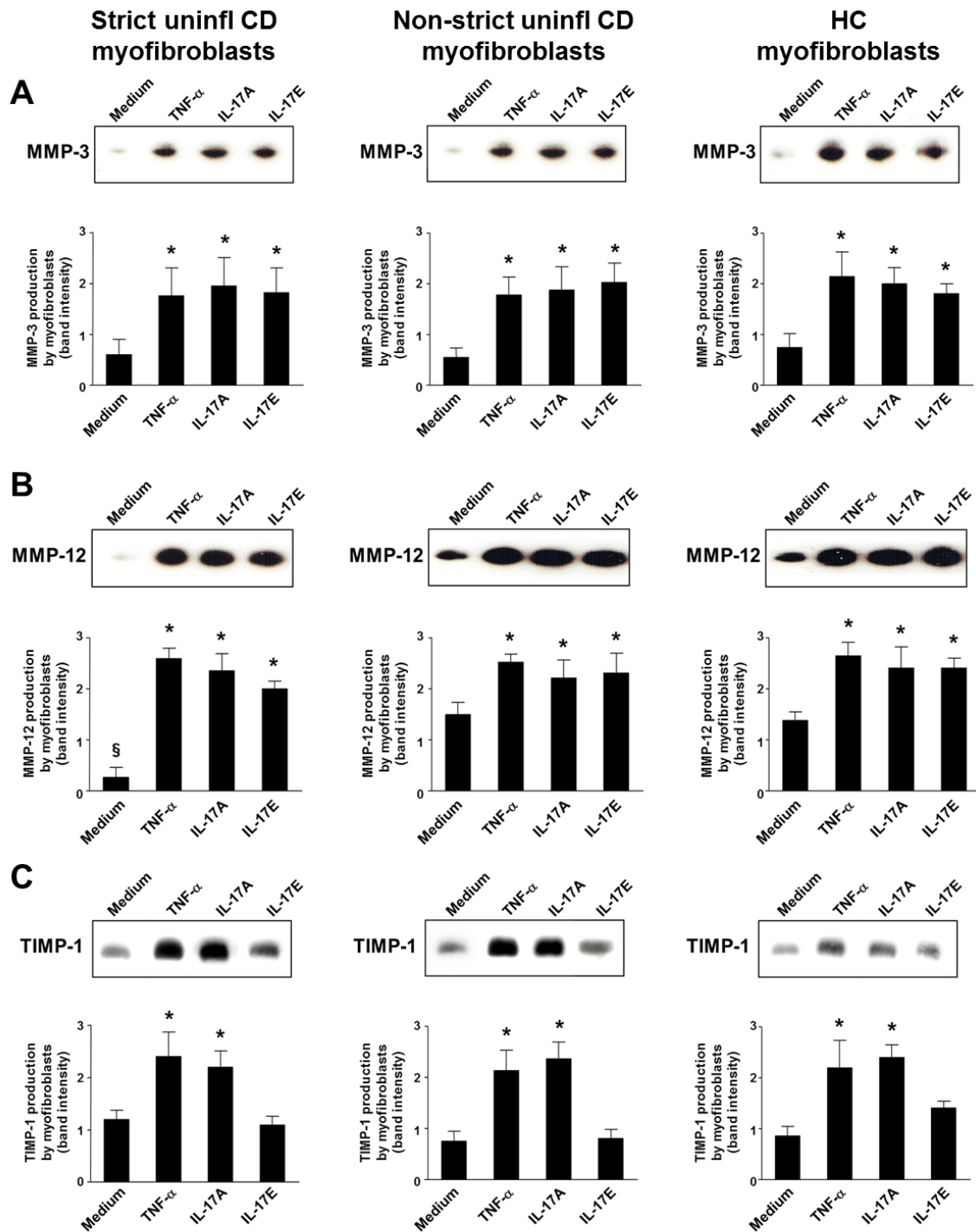


Figure 3.5

Figure 3.5 (see previous page). **Effect of rhIL-17A and rhIL-17E on the production of MMP-3, MMP-12, and TIMP-1 by intestinal myofibroblasts.** (A) MMP-3, (B) MMP-12, and (C) TIMP-1 in culture supernatants of myofibroblasts isolated from uninflamed areas of strictured (Strict uninfl) and non-strictured (Non-strict uninfl) ileum and colon of six patients with fibrostenosing CD and from normal ileum and colon of six control subjects (HCs), cultured for 24 hours with medium alone or rhTNF- α , or rhIL-17A, or rhIL-17E. Each blot displays one experiment, and is representative of experiments performed in all patients with CD and HCs. Lower panels show densitometry of Western blots. Values are mean \pm SEM. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test. * $p < 0.05$ versus unstimulated myofibroblasts. $^{\S}p < 0.05$ versus unstimulated Non-strict uninfl CD and HC myofibroblasts.

3.3.6 *In vitro* effect of rhIL-17A and rhIL-17E on myofibroblast collagen production

Myofibroblasts isolated from uninflamed areas of strictured and non-strictured ileum and colon of six patients with fibrostenosing CD, and from normal ileum and colon of six control subjects were cultured in the presence or absence of rhTNF- α , rhIL-17A, or rhIL-17E, and measured collagen in the culture supernatants (Fig. 3.6). CD myofibroblast stimulation with rhIL-17A induced a significant ($p < 0.05$) increase in mean collagen production (strictured CD myofibroblasts: 412 ± 57 $\mu\text{g/ml}$; non-strictured CD myofibroblasts: 154 ± 17 $\mu\text{g/ml}$) compared to control (myofibroblasts from the same group of patients cultured with medium alone) (strictured CD myofibroblasts: 155 ± 23 $\mu\text{g/ml}$; non-strictured CD myofibroblasts: 76 ± 15 $\mu\text{g/ml}$). Conversely, stimulation with rhIL-17A did not induce a significant increase in collagen production by control myofibroblasts compared to culture with medium alone (114 ± 12 $\mu\text{g/ml}$ and 67 ± 7 $\mu\text{g/ml}$, respectively). Stimulation with rhIL-17E did not induce any significant change in mean collagen production by strictured CD myofibroblasts (181 ± 37 $\mu\text{g/ml}$), non-strictured CD myofibroblasts (83 ± 7 $\mu\text{g/ml}$) or control myofibroblasts (61 ± 10 $\mu\text{g/ml}$) compared to myofibroblasts from the same group of patients cultured with medium alone. Stimulation with rhTNF- α induced a significant

($p < 0.05$) increase in mean collagen production by strictured CD myofibroblasts ($399 \pm 62 \mu\text{g/ml}$), non-strictured CD myofibroblasts ($175 \pm 22 \mu\text{g/ml}$) and control myofibroblasts ($124 \pm 18 \mu\text{g/ml}$) compared with unstimulated cells from the same group of patients. No significant difference in collagen production was found between cells cultured with rhTNF- α and rhIL-17A. Strictured CD myofibroblasts produced significantly ($p < 0.05$) higher amounts of collagen than did non-strictured CD and control myofibroblasts cultured under the same conditions. No significant difference in collagen production was found between non-strictured CD and control myofibroblasts cultured under the same conditions.

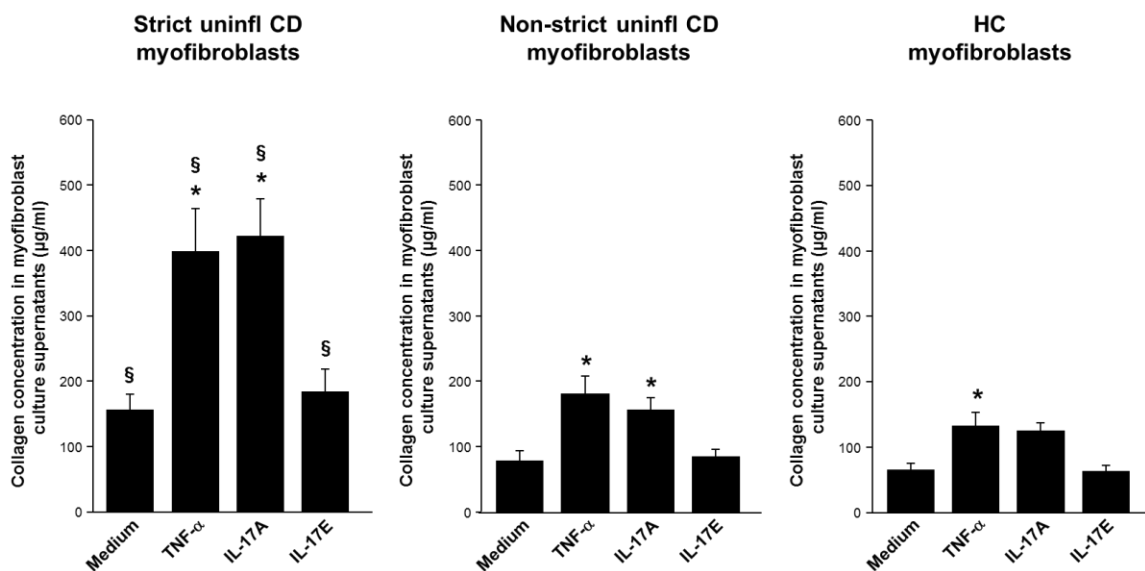


Figure 3.6

Figure 3.6. Effect of rhIL-17A and rhIL-17E on the production of collagen by intestinal myofibroblasts. Levels of collagen, expressed as $\mu\text{g/ml}$, in the supernatants of myofibroblasts isolated from uninflamed areas of strictured (Strict uninfl) and non-strictured (Non-strict uninfl) ileum and colon of six patients with fibrostenosing CD and from normal ileum and colon of six control subjects (HCs), cultured for 24 hours with medium alone or rhTNF- α , or rhIL-17A, or rhIL-17E. Values are mean \pm SEM. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test. * $p < 0.05$ versus unstimulated myofibroblasts. § $p < 0.05$ versus Non-strict uninfl CD and HC myofibroblasts cultured with medium only.

3.3.7 *In vitro* effect of rhIL-17A and rhIL-17E on myofibroblast migration

To evaluate the role of IL-17A and IL-17E on myofibroblast migration, a wound-healing scratch assay was performed using subconfluent monolayers of myofibroblasts isolated from uninflamed areas of strictured and non-strictured ileum and colon of six patients with fibrostenosing CD, and from normal ileum and colon of seven control subjects. Cell migration was measured as the percentage of wound repair and results were expressed as mean percentage of wound repair (Fig. 3.7). rhIL-17A significantly ($p < 0.05$) reduced migration of strictured CD, non-strictured CD and control myofibroblasts at 8 hours ($3.2 \pm 1.1\%$, $4.6 \pm 1.0\%$, and $6.4 \pm 1.6\%$, respectively), 16 hours ($5.8 \pm 1.7\%$, $8.4 \pm 1.8\%$, and $10.6 \pm 1.9\%$, respectively) and 24 hours ($8.8 \pm 1.9\%$, $23.2 \pm 2.3\%$, and $29.7 \pm 2.0\%$, respectively) compared with myofibroblasts from the same three groups cultured with medium alone and evaluated at the same time points (8 hours $9.8 \pm 1.3\%$, $13.7 \pm 1.8\%$, and $14.6 \pm 1.7\%$, respectively; 16 hours: $15.6 \pm 1.6\%$, $25.3 \pm 2.3\%$, and $20.3 \pm 1.1\%$, respectively; and 24 hours: $30.6 \pm 2.3\%$, $49.1 \pm 1.9\%$, and $48.4 \pm 1.8\%$, respectively). Compared with medium alone, rhIL-17E did not induce any significant change in strictured CD, non-strictured CD, and control myofibroblast migration (8 hours: $11.2 \pm 1.2\%$, $15.4 \pm 1.4\%$, $12.9 \pm 1.5\%$, respectively; 16 hours: $16.2 \pm 1.4\%$, $28.2 \pm 1.7\%$, $25.1 \pm 1.1\%$, respectively; and 24 hours: $26.3 \pm 1.9\%$, $41.3 \pm 2.2\%$, $43.4 \pm 2.2\%$, respectively).

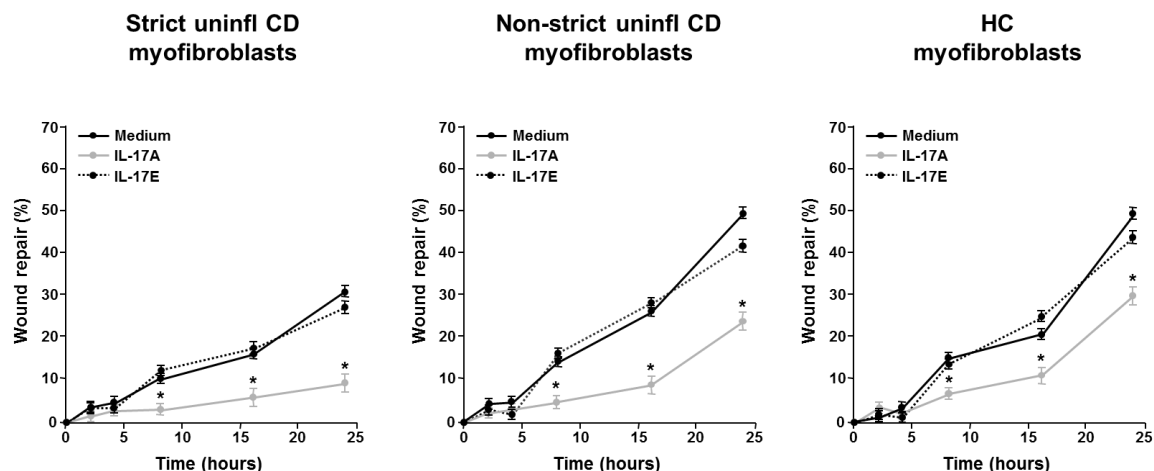


Figure 3.7

Figure 3.7. Effect of rhIL-17A and rhIL-17E on intestinal myofibroblast migration. Effect of rhIL-17A and rhIL-17E on the migration, assessed by an *in vitro* wound-healing scratch assay, of myfibroblasts isolated from uninflamed areas of strictured (Strict uninfl) and non-strictured (Non-strict uninfl) ileum and colon of six patients with fibrostenosing CD and from normal ileum and colon of seven control subjects (HCs). Myfibroblasts were cultured with rhIL-17A or rhIL-17E or medium alone. Results, expressed as percentage of wound repair, are mean \pm SEM. * $p < 0.05$ versus myfibroblasts cultured with medium only at 8, 16, and 24 hours.

3.4 Results summary

In the current study, we found that:

1. IL-17A, but not IL-17E, is overexpressed in strictured CD ileum and colon compared with non-strictured CD and control ileum and colon.
2. Intestinal tissue explants from strictured CD ileum cultured *ex vivo* release significantly more IL-17A, but not IL-17E, compared with intestinal tissue explants from non-strictured CD and control ileum.
3. Strictured CD, non-strictured CD, and control ileum and colon, and intestinal myofibroblasts from CD and control ileum and colon express both IL-17RC and IL-17RB, without significant differences between the groups studied.
4. Stimulation with IL-17A significantly up-regulates MMP-3, MMP-12, TIMP-1 and collagen release by intestinal myofibroblasts from CD and control ileum and colon cultured *in vitro*, whereas it significantly down-regulates the migratory ability of intestinal myofibroblasts from CD and control ileum and colon cultured *in vitro*. Stimulation with IL-17E significantly up-regulates MMP-3 and MMP-12, but not TIMP-1 and collagen release by intestinal myofibroblasts from CD and control ileum and colon cultured *in vitro*, and it has no significant effect on the migratory ability of intestinal myofibroblasts from CD and control ileum and colon cultured *in vitro*.

3.5 Discussion

Our findings indicate that IL-17A, but not IL-17E, expression is up-regulated in fibrostenosing CD intestine, and that IL-17A, but not IL-17E, may contribute to intestinal fibrosis in CD. This Section focuses on the interpretation of our results in the context of the existing literature, on the limitations of our study, and on possible future experiments aimed at clarifying further the role of IL-17 in CD intestinal fibrosis.

IL-17A and IL-17E expression in intestinal fibrosis

IL-17A, which is produced by several cell types including T cells, NKT cells and neutrophils, can exert several pro-fibrogenic actions (Meng *et al.* 2012; Okamoto *et al.* 2012; Valente *et al.* 2012). In particular, it has been shown that *in vitro* stimulation with IL-17A enhances the expression of pro-fibrogenic mediators, such as TGF- β and type I collagen, by pulmonary fibroblasts in bleomycin-induced systemic sclerosis, and this effect is inhibited by anti-IL-17 treatment (Lei *et al.* 2016). Moreover, IL-17A produced by $\gamma\delta$ T cells is required for inflammation and destruction of the biliary system in a mouse model of virus-induced biliary atresia (Klemann *et al.* 2016). IL-17A plays an important role in liver fibrosis by promoting hepatic stellate cell activation, and defective IL-17 signalling has a protective effect on experimental cholestatic liver fibrosis (Meng *et al.* 2012; Hara *et al.* 2013; Tan *et al.* 2013). It has also been shown that treatment with an anti-IL-17A monoclonal antibody attenuates liver fibrosis following bile duct ligation (Zhang *et al.* 2016).

IL-17E is produced by various cell types, including T cells, epithelial cells and eosinophils, and has a well-established role in allergic diseases including asthma, and in the prevention of helminth infections (Iwakura *et al.* 2011). In the human intestinal mucosa, IL-17E is produced by subepithelial macrophages (Caruso *et al.* 2009), which may be involved in intestinal fibrosis. IL-17E has been shown to

promote Th2 and to suppress Th1 and Th17 immune responses, possibly by inhibiting the expression of IL-12 and IL-23 (Sharkhuu *et al.* 2006; Caruso *et al.* 2009). IL-17E has known pro-fibrogenic properties, especially in the context of experimental pulmonary fibrosis (Létuvé *et al.* 2006; Gregory *et al.* 2013). On the other hand, the administration of IL-17E has been shown to ameliorate bile duct ligation-induced liver fibrosis in mice (Meng *et al.* 2012), suggesting that the inhibitory effect of IL-17E on Th17 cell development is predominant in this model.

In the present study, we observed that IL-17A, but not IL-17E, is overexpressed in strictured CD ileum and colon compared with non-strictured CD and control ileum and colon. We collected samples from uninfamed areas of patients with fibrostenosing CD, in order to minimise any confounding effect of inflammation on IL-17A. It has been observed that IL-17A expression is higher in long-standing CD mucosa compared with early mucosal lesions (Zorzi *et al.* 2013), which is compatible with the possible involvement of IL-17A in CD intestinal fibrosis, as this latter is a late-stage process in CD.

While ours is the first report on mucosal levels of IL-17 in fibrostenosing CD, there are data in the literature on intestinal IL-17 expression in experimental intestinal fibrosis. Mucosal up-regulation of both IL-17A and IL-17E has been reported to occur approximately 21 days after the first TNBS administration in chronic TNBS-induced colitis, which is associated to the development of intestinal fibrosis. Interestingly, mucosal IL-17E production continued to increase even after the levels of IL-17A reached a plateau, and was associated with the up-regulation of IL-13. In this particular model, collagen up-regulation occurs between day 35 and day 42, therefore both IL-17A and IL-17E overexpression appear to precede the onset of fibrosis (Fichtner-Feigl *et al.* 2008b). The functional relevance of the data on IL-17 family members in experimental intestinal fibrosis is still unclear. However, when considering the time of onset of the up-regulation of the different cytokines, one possible interpretation is that IL-17E, which does not appear to influence the simultaneous IL-17A

overexpression, may contribute to Th2 cell development with consequent overexpression of IL-13, and that IL-17A up-regulation may exert a pro-fibrogenic action via an independent pathway.

Functional role of IL-17A and IL-17E in intestinal fibrosis

It is difficult to determine whether the IL-17A up-regulation that we observed in CD strictures is an epiphenomenon due to the persistence of a pre-existing inflammatory infiltrate, and therefore IL-17A is a by-stander in the process of fibrosis, or whether this cytokine plays a functional role in stricture formation *in vivo*. It is difficult to solve this problem using human tissue, since intestinal strictures in CD are the end-stage product of a long-standing process. Furthermore, there are no clear predictors of intestinal fibrosis in CD (Rieder *et al.* 2014), which would allow the selection of a subset of patients to be studied prospectively in order to elucidate the relative contribution of different types of immune response and cytokines in the early phases of stricture development.

The ideal tool to study the early phases of intestinal fibrosis would be an animal model with similar features to CD. Unfortunately, however, currently existing mouse models of intestinal fibrosis, such as chronic TNBS- and DSS-induced colitis, have few similarities to CD. One of the most relevant differences is that in the experimental model, unlike in CD, the causative agent of fibrosis is a pre-determined chemical agent. Novel models are emerging, which are more similar to CD. One of them is the AIEC-induced colitis, in which mice, upon pre-treatment with streptomycin, receive by gavage the AIEC NRG857 strain, isolated from the intestinal microbiota of CD patients, and as a result, they develop Th1-/Th17-mediated intestinal fibrosis (Small *et al.* 2013). This model has the advantage of using a microbial strain which has been found preferentially in the intestine of CD patients, however the degree of intestinal fibrosis is only mild. Another interesting mouse model to study the development and progression of intestinal fibrosis is represented by the SAMP mice, which spontaneously

develop terminal ileitis and subsequently fibrosis, and are characterised by impaired migration of DCs to MLNs, suggesting a defect in immunologic tolerance (Mikulski *et al.* 2015). It has been shown that the mucosal immune response in the early phases of intestinal inflammation in SAMP mice is characterised predominantly by a Th1 profile, which is subsequently replaced by a Th2 profile when fibrosis is established, however the role of IL-17 in this model has never been investigated (Ray *et al.* 2014).

We have attempted to investigate the possible functional role of IL-17A in intestinal fibrosis by means of *in vitro* experiments using intestinal myofibroblasts. In keeping with the study of Bamba *et al.* (Bamba *et al.* 2003), we found that IL-17A stimulation *in vitro* increases MMP-3 and MMP-12 production by myofibroblasts from strictured CD, non-strictured CD and control ileum and colon. In parallel, we observed that IL-17A up-regulates TIMP-1 and collagen release, and reduces the migration ability of CD and control myofibroblasts. Our results have been confirmed by Honzawa *et al.* (Honzawa *et al.* 2014), who showed that IL-17A promotes collagen I expression by intestinal subepithelial myofibroblasts from IBD patients via heat shock protein (HSP)47, and that HSP47 knock-down by mRNA interference in intestinal subepithelial myofibroblasts abrogates IL-17A-induced collagen I up-regulation. Collectively, our *in vitro* data suggest that IL-17A has a complex effect on the process of intestinal fibrosis, as the up-regulation in tissue-degrading MMP-3 and MMP-12 is counterbalanced by the up-regulation of TIMP-1. Based on our *in vitro* data, however, the overall effect of IL-17A is more likely to be pro-fibrogenic, as suggested by the up-regulation of collagen release and the reduction in the migratory ability. The stimulation with IL-17E increases MMP-3 and MMP-12 production by myofibroblasts from CD and control ileum and colon, however we did not observe any influence of IL-17E on collagen or TIMP-1 production by CD myofibroblasts, or on their migratory ability. Based on the expression data and on the *in vitro* results, it is therefore difficult to imagine a prominent role of IL-17E in CD intestinal fibrosis.

Collectively, both the data present in the literature and our data suggest that IL-17 family member, and more generally Th17 cells, play a complex role in intestinal inflammation and fibrosis. An example of this is represented by the observations that *in vitro* stimulation of CD myofibroblasts with an agonist of aryl hydrocarbon receptor (AhR), which is essential for Th17 cell differentiation and activation, induces the down-regulation of collagen synthesis, and that treatment with the same AhR agonist ameliorates TNBS-induced intestinal fibrosis (Monteleone *et al.* 2016).

Limitations of our study

In our study, we analysed the intestinal expression of IL-17 family members and IL-17Rs using Western blotting and ELISA assays, however other methods such as qRT-PCR and immunohistochemistry would be useful to corroborate our data, and should be used in the first part of a validation study. Moreover, the magnitude of IL-17A up-regulation which we observed in CD strictures compared with non-strictured CD areas and control intestine is in the range of a 2-3 fold factor, which raises the question whether this statistically significant difference has a biological relevance in CD intestinal fibrosis.

Another limitation of our study is represented by the choice of ECM components and tissue remodelling enzymes which we used as read-outs to assess the pro-fibrogenic potential in our *in vitro* experiments. We chose to evaluate the production of MMP-3, MMP-12 and TIMP-1 by intestinal myofibroblasts because of the prominent and established involvement in IBD pathogenesis, or because of existing data in the same context and experimental model, or because of the availability of the reagents and of an optimised detection protocol (Di Sabatino *et al.* 2009a). It would be appropriate to study the effects of IL-17A and IL-17E on the production of other proteases and their inhibitors, such as MMP-9, MMP-13 and TIMP-3, however we decided not to study MMP-7 as fibrosis occurs in the submucosal and muscle layers, and we focused on these layers upon discarding

the mucosa of the intestinal samples. Nevertheless, the measurement of total collagen concentration in myofibroblast supernatants and myofibroblast migratory ability provides the evaluation of downstream events in the fibrogenic cascade, which may be more representative of the *in vivo* conditions than upstream mediators such as proteases and their inhibitors.

Future experiments

In addition to the experiments mentioned in the paragraph about study limitations, such as the measurement of intestinal IL-17 family member expression by qRT-PCR and immunohistochemistry, and the evaluation of other pro-fibrogenic mediators, among which fibronectin and collagen subtypes appear to be particularly relevant, it would be important to assess the expression of other IL-17 family members and Th17 cytokines, including IL-17F and IL-22, in the context of CD intestinal fibrosis.

An important point still to be clarified is the evaluation of the mucosal expression levels and the contribution of different IL-17 family members to intestinal fibrosis during the phases that precede the onset of CD strictures. The first step in order to address this question could involve using AIEC-induced colitis and SAMP mice, which are currently the experimental models of intestinal fibrosis most closely related to CD.

It has been shown that cathelicidin, an anti-microbial peptide with anti-inflammatory properties, can reverse TNBS-induced intestinal fibrosis by inhibiting collagen production by colonic fibroblasts via a MAPK-dependent mechanism (Yoo *et al.* 2015). Additionally, cathelicidin down-regulates IL-17A and up-regulates TGF- β production by monocyte-derived macrophages infected with *M. tuberculosis* (Torres-Juarez *et al.* 2015). It is known that AIEC NRG857 strain has a pro-fibrogenic effect in experimental colitis, that anti-microbial peptides, albeit via their anti-inflammatory action, have an anti-fibrogenic effect,

and that components of the intestinal microbiota such as SFB have important effects on Th17 cells. Hence, it would be particularly interesting to investigate the effects of specific components of the intestinal microbiota and anti-microbial peptides on the mechanisms of intestinal fibrosis, with particular reference to the Th17 immune response. In the first instance, this could be done by characterising the intestinal microbiota of patients with fibrostenosing CD compared to non-fibrostenosing CD patients, and subsequently by studying the *in vitro* effects of differentially represented species on the mechanisms of intestinal fibrosis.

Finally, it will be important to clarify which cell types are the main responsible for the overproduction of specific Th17-related cytokines at different time points during intestinal stricture development in CD. A starting point to address this question will be to perform intracellular flow cytometry on freshly isolated cells from fibrostenosing CD patients, and to determine which cell types are responsible for the overproduction of IL-17A in this condition. Interestingly, it has been shown that, among Th17 cells, upon TCR stimulation, only a sub-population with stable expression of multi-drug resistance type 1 (MDR1), but not their MDR1⁻ counterparts, can produce IL-17A and IFN- γ , and no IL-10 or other anti-inflammatory molecules. MDR1⁺ Th17 cells are enriched in the intestinal mucosa of CD patients (Ramesh *et al.* 2014), hence in a subsequent validation study it would be appropriate to assess whether this cell population is involved in CD intestinal fibrosis.

Chapter 4: The role of IL-13 in IBD

Note: The quantification of NKT, iNKT and Th2 cells within intestinal lamina propria and the evaluation of IL-13 production by mucosal NKT cells (Paragraphs 4.3.2-4) have been performed by Dr F Facciotti in the Laboratories of the Istituto Nazionale di Genetica Molecolare, Milan, Italy. Primary data of the experiment are not available, therefore I could not repeat the statistical analysis using a different and more appropriate test, such as the ANOVA One way analysis of variance. These results have been published in the following paper, on which I am first Author (Biancheri P, Di Sabatino A, Ammoscato F, Facciotti F, Caprioli F, Curciarello R, Hoque SS, Ghanbari A, Joe-Njoku I, Giuffrida P, Rovedatti L, Geginat J, Corazza GR, MacDonald TT. Absence of a role for interleukin-13 in inflammatory bowel disease. *Eur J Immunol* 2014;44:370-85). Substantial help in the study of IL-4R and IL-13R expression by intestinal T cells, macrophages and myofibroblasts (Paragraphs 4.3.9-12) has been provided by Ms F Ammoscato in the Centre for Immunobiology Laboratory, Blizard Institute, Barts and The London School of Medicine and Dentistry, London, UK.

IL-13 has been reported to be up-regulated in UC intestinal mucosa, and to play an important role in oxazolone-induced colitis and in intestinal experimental fibrosis. However, contrasting observations exist on the levels and the role of IL-13 in inflamed IBD mucosa, and limited information is available on the role of IL-13 in CD intestinal fibrosis. Further information on the background to this Chapter is reported in Sections “1.4.4 Inappropriate inflammatory reaction in IBD intestinal mucosa” and “1.4.7 Mechanisms of intestinal fibrogenesis in CD”.

4.1 Aims of the study

This study aims to explore the role of IL-13 in UC mucosal inflammation and in CD intestinal fibrosis using the following approach:

5. Assessment of IL-13 production by mucosal intestinal explants and LPMCs

from UC patients, CD patients and control subjects.

6. Evaluation of IL-13-producing cells in the intestinal mucosa of UC patients, CD patients and control subjects.
7. Assessment of IL-13 production by intestinal tissue explants from fibrostenosing CD patients and control subjects.
8. Assessment of IL-13R expression on mucosal T cells and macrophages from UC patients, CD patients and control subjects.
9. Assessment of IL-13R expression on submucosal myofibroblasts of CD patients and control subjects.
10. Study of the *in vitro* effects of IL-13 on UC LPMCs.
11. Study of the *in vitro* effects of IL-13 on CD and control intestinal myofibroblasts.

4.2 Chapter-specific Materials and Methods

4.2.1 Patients and tissues

Colonic biopsies or surgical specimens were taken from macroscopically and microscopically inflamed or uninflamed mucosa of 44 patients affected by CD (Table 4.1) and 51 patients affected by UC (Table 4.2). Surgical specimens were also taken from uninflamed areas of strictured and non-strictured ileum of 13 patients with fibrostenosing CD (Table 4.1). CD and UC patients were enrolled consecutively. Diagnosis of CD and UC was made according to standard clinical criteria (Van Assche *et al.* 2010), and the site and extent of the disease were confirmed by endoscopy. In CD patients, disease activity was assessed by Crohn's Disease Activity Index (Best *et al.* 1976). Patients with scores below 150 were classified as being in remission, whereas those with scores over 450 had severe disease (Best *et al.* 1976). In UC patients, disease activity was assessed according to the Clinical Activity Index (Rachmilewitz 1989). Clinical remission was defined as a score below 4. None of the IBD patients had been ever treated with cyclosporine, tacrolimus, methotrexate or anti-TNF- α antibodies. Mucosal samples were collected perendoscopically from the ileum or the colon of 22 subjects who had functional diarrhoea at the end of their diagnostic work-up (Table 4.3), who were considered as control subjects. Intestinal samples were also collected from macroscopically and microscopically unaffected (at least 500 mm far from the tumour mass) ileal or colonic areas of 17 patients undergoing intestinal resection for colon cancer (Table 4.4), who were considered as control subjects. CD, UC patients and control subjects who provided intestinal perendoscopic biopsies were sex-matched, whereas control subjects who underwent intestinal resection were significantly ($p < 0.001$) older than CD and UC patients and than control subjects who provided intestinal perendoscopic biopsies. This study was approved by the NRES Committee London - City & East (REC reference: 10/H0704/74), and informed consent was obtained from all patients and control subjects.

Table 4.1. Clinical features of patients with CD (n=57)

Characteristics and parameters	N.	Median (range)
Age (years)		32.5 (18-61)
Sex (M/F)	31/26	
First attack	14	
Intestinal location		
<i>Small bowel and colon</i>	41	
<i>Colon only</i>	16	
Disease behaviour		
<i>Fistulising</i>	8	
<i>Fibrostenosing</i>	13	
<i>Luminal</i>	36	
Duration of disease (months)		47.3 (1-188)
Number of recurrences		3 (0-9)
CDAI		215 (73-412)
Treatment		
<i>Mesalazine</i>	42	
<i>Topical steroids</i>	15	
<i>Antibiotics</i>	12	
<i>Azathioprine/6-MP</i>	16	

6-MP, 6-mercaptopurine.

Table 4.2. Clinical features of patients with UC (n=51)

Characteristics and parameters	N.	Median (range)
Age (years)		34.5 (17-56)
Sex (M/F)	24/27	
First attack	6	
Intestinal location		
<i>Pancolitis</i>	21	
<i>Left-sided colitis</i>	30	
Duration of disease (months)		48.8 (1-173)
Number of recurrences		3.5 (0-6)
CAI		8 (5-13)
Treatment		
<i>Mesalazine</i>	10	
<i>Mesalazine + topical steroids</i>	22	
<i>Mesalazine + azathioprine/6-MP</i>	12	

6-MP, 6-mercaptopurine; CAI, Clinical activity index.

Table 4.3. Clinical features of control subjects who provided intestinal perendoscopic biopsies (n=22)

Characteristics and parameters	N.	Median (range)
Age (years)		38 (25-62)
Sex (M/F)	12/10	
Comorbidities		
<i>Hypertension</i>	4	
<i>Hyperlipaemia</i>	4	
<i>Diabetes</i>	3	
<i>Chronic obstructive lung disease</i>	1	
<i>Chronic kidney disease</i>	1	
<i>None</i>	13	
Current medications		
<i>Anti-hypertensive</i>	4	
<i>Diuretics</i>	2	
<i>Aspirin</i>	2	
<i>Statins</i>	3	
<i>Metformin</i>	3	
<i>Bronchodilators</i>	1	
<i>None</i>	10	

F, female; M, male.

Table 4.4. Clinical features of control subjects who underwent intestinal resection (n=17)

Characteristics and parameters	N.	Median (range)
Age (years)		57 (42-72)
Sex (M/F)	9/8	
Comorbidities		
<i>Ischaemic heart disease</i>	3	
<i>Hypertension</i>	6	
<i>Hyperlipaemia</i>	8	
<i>Diabetes</i>	2	
<i>None</i>	13	
Current medications		
<i>Anti-hypertensive</i>	6	
<i>Diuretics</i>	1	
<i>Aspirin</i>	3	
<i>Statins</i>	5	
<i>Metformin</i>	2	
<i>None</i>	8	

F, female; M, male.

4.2.2 Study design

In order to study the involvement of IL-13 in intestinal inflammation, spontaneous IL-13 and pro-inflammatory cytokine production by intestinal biopsies from 10 CD patients, 13 UC patients and 10 control subjects cultured *ex vivo* was evaluated (Fig. 4.1, Fig. 4.2). Additionally, the production of IL-13 and pro-inflammatory cytokines by unstimulated or activated intestinal LPMCs isolated from additional 15 CD patients, 15 UC patients and 15 control subjects was studied (Fig. 4.9, Fig. 4.10, Fig. 4.11, Table 4.5). The expression of IL-13 and other pro-inflammatory cytokines was studied in biopsy homogenates from additional nine CD patients, additional seven UC patients and 16 of the aforementioned control subjects (Fig. 4.12). The distribution of NKT cells and their production of IL-13 were evaluated in LPMCs from the inflamed intestinal mucosa of additional four CD patients, seven UC patients, and in the normal intestinal mucosa of additional three control subjects (Fig. 4.4, Fig. 4.8). Moreover, the distribution of iNKT and Th2 cells was evaluated in LPMCs from the inflamed intestinal mucosa of additional three CD patients and three UC patients, and from the normal intestinal mucosa of additional three control subjects (Fig. 4.6). The expression of IL-13Rs and IL-4R α by LPMCs isolated from the inflamed colon of additional three CD patients and three UC patients, and from the normal colon of additional three control subjects was then characterised (Fig. 4.15, Fig. 4.16, Fig. 4.18, Fig. 4.19). Finally, the effects of IL-13 or IL-13R blockade on LPMCs isolated from the inflamed colon of three additional UC patients were evaluated (Fig. 4.22, Fig. 4.24).

In order to study the role of IL-13 in CD fibrosis, spontaneous IL-13 and pro-fibrogenic mediator production by muscle layer explants from the ileum of six fibrostenosing CD patients, five additional control subjects and two of the aforementioned control subjects was evaluated (Fig. 4.13). The expression of IL-13Rs and IL-4R α by LPMCs isolated from the ileum of three additional fibrostenosing CD patients was then characterised (Fig. 4.15, Fig. 4.16, Fig. 4.18, Fig. 4.19). Ileal submucosal myofibroblasts from four additional fibrostenosing

CD patients, from one of the aforementioned fibrostenosing CD patients and from five of the aforementioned control subjects were isolated and, after characterising the expression of IL-13Rs and IL-4R α (Fig. 4.21), were cultured with IL-13 and the production of collagen in the supernatant was measured (Fig. 4.25).

4.2.3 LPMC isolation and culture

LPMCs were isolated as follows within 30 minutes from biopsy/specimen collection. The epithelial layer was removed from the lamina propria with 1 mM EDTA (Sigma-Aldrich) treatment. After stirring for 1 hour at 37°C, the supernatant was removed and the remaining tissue was treated with type 1A collagenase (1 mg/ml; Sigma-Aldrich) for 1 hour with stirring at 37°C. The crude cell suspension was allowed to stand for 5 minutes to permit debris sedimentation. Cells from the supernatant were washed twice, resuspended in 1 ml RPMI-1640 medium (Sigma-Aldrich) containing 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin, and kept on ice until used. Cells were not used if viability, as assessed by flow cytometry after propidium iodide staining, did not exceed 90%. Freshly isolated LPMCs were cultured (2×10^5 cells/well, in duplicate) in RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin, at 37°C, 5% CO₂ for 48 hours in 96-well plates (VWR International) with medium alone or with rhIL-13 (PeproTech EC, London, UK), or in anti-CD3-coated 96-well plates (BD Biosciences) with soluble anti-CD28 antibody (1 μ g/ml; eBioscience), in the absence or presence of the following stimuli: rhIL-13, polyclonal goat anti-IL-13R α 2 neutralising antibody (R&D Systems, goat IgG), monoclonal mouse anti-IL-4 receptor (IL-4R) α neutralising antibody (R&D Systems, Mouse IgG_{2a}), goat IgG (Sigma-Aldrich), mouse IgG_{2a} (R&D Systems), soluble anti-CD2 (1 μ g/ml; BioLegend, Cambridge, UK) and soluble anti-CD28 antibody (1 μ g/ml; eBioscience). All the above mentioned antibodies and control IgG were used at a concentration of 10 μ g/ml, while rhIL-13 was used at 20 ng/ml. The total processing time from collection to cell resuspension was less than 3 hours. After culture, LPMC supernatants were

collected and stored at -70°C. For the purposes of analysis of IL-13 production by flow cytometry, LPMCs were cultured with immobilised anti-CD3 antibodies (2 µg/ml) in the presence of 2 µg/ml anti-CD28 antibodies (BD Biosciences) for 44 hours before addition for 4 hours of brefeldin A (Sigma-Aldrich).

4.2.4 Flow cytometry

LPMCs and myofibroblasts were stained with the following antibodies (or corresponding isotype control antibodies): FITC-anti-human CD3 (UCHT1, BD Biosciences; Mouse IgG₁(κ) isotype control), APC-anti-human CD4 (RPA-T4, BD Biosciences, Mouse IgG₁(κ) isotype control; VIT4, Miltenyi Biotec, Mouse IgG_{2a}(κ) isotype control), PE-anti-human CD294 (CRTH2) (BM16, Miltenyi Biotec, Rat IgG_{2a}(κ) isotype control), APC-anti-human CD161 (DX12, BD Biosciences, Mouse IgG₁(κ) isotype control), APC-anti-human CXCR3 (1C6, BD Biosciences, Mouse IgG₁(κ) isotype control), PE/Cy7-anti-human CD127 (HTK888, BioLegend, Rat IgG_{2a}(κ) isotype control), FITC-anti-human lineage cocktail (UCHT1, BioLegend, Mouse IgG₁(κ) isotype control), PE-anti-human Vα24 (6B11, BioLegend, Mouse IgG₁(κ) isotype control), APC-anti-human Vβ11 (C21, Becton Dickinson, Mouse IgG_{2a}(κ) isotype control), APC-anti-human IL-4Rα (FAB230A, R&D Systems, Mouse IgG_{2a}(κ) isotype control), Alexa Fluor[®] 700-anti-human IL-13Rα1 (FAB1462N, R&D Systems, Mouse IgG_{2a}(κ) isotype control), fluorescein-anti-human IL-13Rα2 (FAB614F, R&D Systems, Mouse IgG_{2b}(κ) isotype control), biotinylated anti-human CD68 (Y1/82A, BioLegend, Mouse IgG_{2b}(κ) isotype control), PE-anti-human IL-13 (JES10-5A2, BD Biosciences, Rat IgG₁ isotype control). Biotinylated monoclonal antibodies were revealed with Streptavidin-APC-Cy7 (BD Biosciences). Samples were passed on a FACSCanto II flow cytometer (BD Biosciences). A minimum of 30,000 events/test tube for LPMC stainings, and a minimum of 10,000 events/test tube for myofibroblasts were collected. The doublets corresponding to cell aggregates were excluded by gating, and single cells were further analysed. Events were also gated to exclude

nonviable cells on the basis of light scatter, and to eliminate artefacts caused by poor flow. Gates were set up according to the isotype staining profiles, and differences in setting the gates for the same staining between different samples analysed are attributable to interindividual variability. Data were analysed using FlowJo software (Treestar, Ashland, OR).

4.2.5 T84 cell culture

Subconfluent monolayers of T84 cells were cultured in 24-well plates (VWR International) for 72 hours at 37°C, 5% CO₂, with DMEM/Hams Nutrient F12 Mix (Invitrogen Ltd), supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine (Invitrogen Ltd), in the absence or presence of the following stimuli: rhIL-13 (PeproTech EC), polyclonal goat IL-13R α 2 neutralising antibody (R&D Systems), monoclonal mouse anti-IL-4R α neutralising antibody (R&D Systems), goat IgG (Sigma-Aldrich), mouse IgG_{2a} (R&D Systems). All the above mentioned antibodies and control IgG were used at a concentration of 10 µg/ml, while rhIL-13 was used at 20 ng/ml.

4.3 Results

4.3.1 *Ex vivo* production of IL-13, IFN- γ , IL-17A, IL-4 and IL-5 by mucosal biopsies

The spontaneous production of IL-13, IL-17A, IFN- γ , IL-4 and IL-5 by biopsies in organ culture from inflamed and uninfamed mucosa of 23 IBD patients (10 with CD and 13 with UC) and 10 control subjects was determined (Fig. 4.1 and 4.2).

The concentration of IL-13 was the same in organ culture supernatants of IBD inflamed mucosa (CD: median 44 pg/ml, range 18-77; UC: median 37 pg/ml, range 0-96), IBD uninfamed mucosa (CD: median 39 pg/ml, range 15-62; UC: median 35 pg/ml, range 12-72), and control mucosa (median 31 pg/ml, range 10-71) (Fig. 4.1). IL-13 measurement on the same supernatants was repeated using a different and more sensitive kit, and in this case IL-13 was actually undetectable in all the supernatants tested.

Since it is well known that IL-17A and IFN- γ are elevated in CD and UC, these cytokines were also measured in the same supernatants (Fig. 4.1). IFN- γ was significantly higher in the supernatants of inflamed CD (median 235 pg/ml, range 72-465; $p < 0.001$) and inflamed UC (median 210 pg/ml, range 37-370; $p < 0.01$) biopsies compared with those of control subjects (median 59 pg/ml, range 25-82). The concentration of IL-17A was significantly higher in the supernatants of inflamed CD (median 164 pg/ml, range 52-467; $p < 0.001$) and inflamed UC (median 171 pg/ml, range 68-434; $p < 0.01$) organ culture biopsies in comparison with those of uninfamed areas (CD: median 44 pg/ml, range 20-98; UC: median 72 pg/ml, range 38-150) and control subjects (median 55 pg/ml, range 31-93; $p < 0.01$).

IL-4 and IL-5 were not detectable in all supernatants of inflamed and uninfamed IBD and control mucosa (Fig. 4.2).

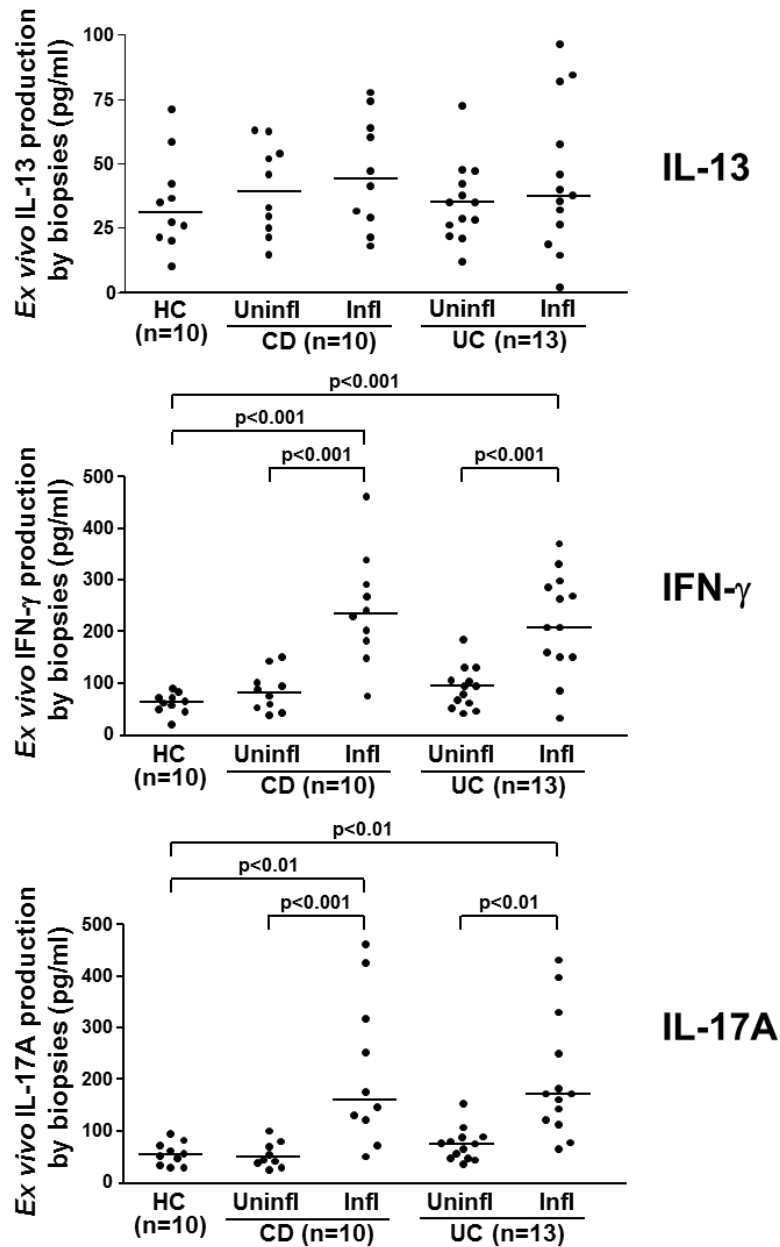


Figure 4.1

Figure 4.1. Ex vivo production of IL-13, IFN- γ and IL-17A by intestinal biopsies. Levels of IL-13, IFN- γ and IL-17A expressed in pg/ml, in the supernatants of intestinal biopsies taken from the inflamed and uninflamed mucosa of 10 CD patients and 13 UC patients, and from the mucosa of 10 control subjects (HC), and cultured for 24 hours in the absence of stimuli. Each point represents one patient. Horizontal bars are median values. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test.

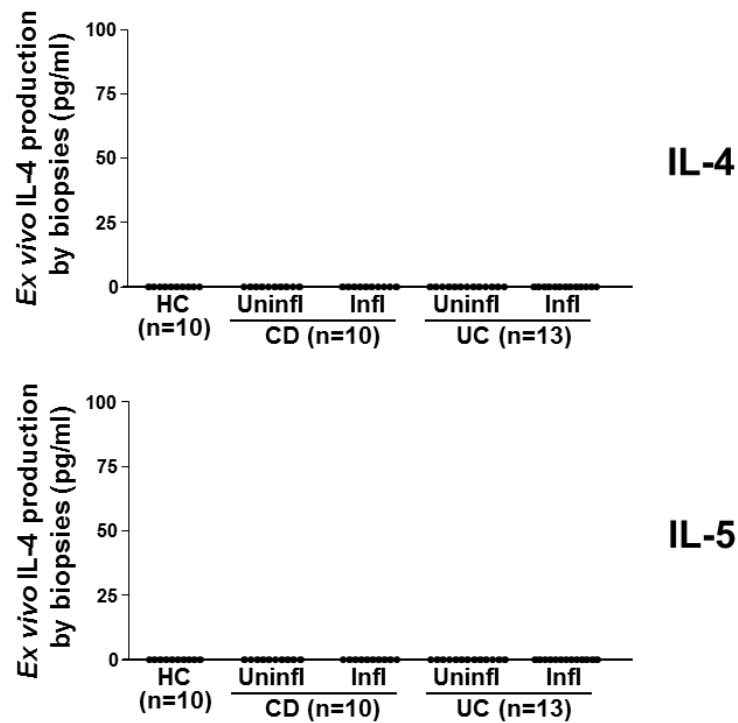


Figure 4.2

Figure 4.2. *Ex vivo* production of IL-4 and IL-5 by intestinal biopsies. Levels of IL-4 and IL-5 expressed in pg/ml, in the supernatants of intestinal biopsies taken from the inflamed and uninflamed mucosa of 10 CD patients and 13 UC patients, and from the mucosa of 10 control subjects (HC), and cultured for 24 hours in the absence of stimuli. Each point represents one patient. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test.

4.3.2 Relative distribution of NKT cells among T-LPLs

Previous studies showed that NKT cells, iNKT cells and Th2 lymphocytes are among the most relevant producers of IL-13 in humans (van Dieren *et al.* 2007). Flow cytometry was used to determine the distribution of NKT cells, defined as CD3⁺ cells expressing CD161 (please see gating strategy in Fig. 4.3), among sorted T-LPLs from normal intestinal mucosa of three control subjects and from inflamed intestinal mucosa of four clinically active patients with CD and seven clinically active patients with UC (Fig. 4.4). NKT cells were significantly ($p < 0.01$) reduced in UC (mean $12.02 \pm 4.39\%$) compared with control (mean $69.33 \pm 4.90\%$) and CD T-LPLs (mean $38.00 \pm 16.19\%$). The percentage of NKT cells did not differ significantly between control and CD T-LPLs.

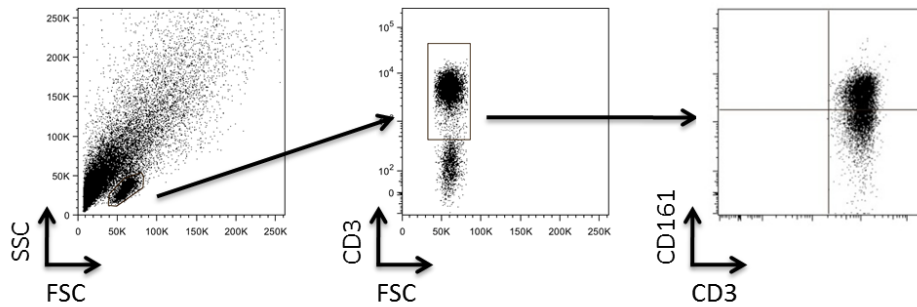


Figure 4.3

Figure 4.3. Gating strategy used for the evaluation of NKT cell distribution in the intestinal lamina propria. LPMCs were isolated from the intestinal mucosa of IBD patients and control subjects, and were stained with FITC-anti-human CD3 and APC-anti-human CD161. Subsequently, cells were analysed by flow cytometry. NKT cells were identified as CD161⁺ (gated on viable CD3⁺ cells). The dot plot displays one experiment, and is representative of experiments performed in all patients with IBD and control subjects.

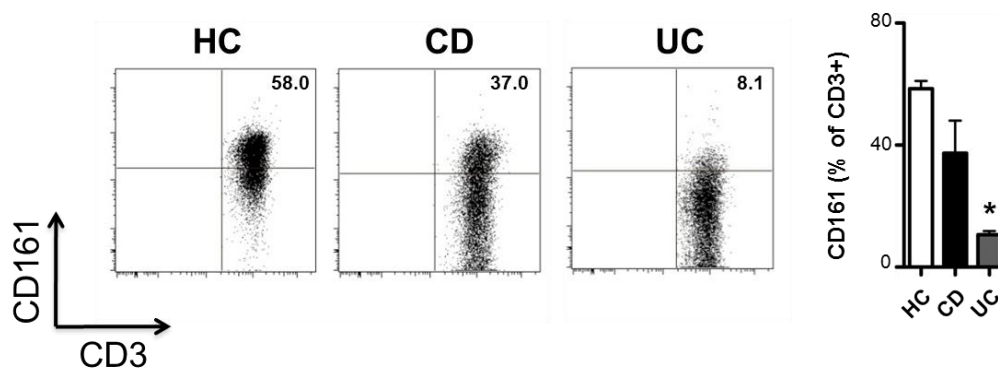


Figure 4.4

Figure 4.4. NKT cell distribution in the intestinal lamina propria. Relative distribution of NKT cells in T-LPLs of control subjects (HC, n=3) and patients with CD (n=4) and UC (n=7). NKT cells were identified as CD161⁺ (gated on viable CD3⁺ cells). Each dot plot displays one experiment, and is representative of experiments performed in all patients with IBD and control subjects. Right histogram bar graph: mean percentage of NKT cells out of total viable T-LPLs from HC subjects and CD and UC patients. Bars represent mean \pm SEM. The Mann-Whitney *U* test was applied. * $p < 0.01$ versus HC and CD T-LPLs.

4.3.3 Relative distribution of iNKT and Th2 cells among T-LPLs

Similarly, the percentage of iNKT cells, defined as CD3⁺ cells that express a semi-invariant TCR composed in humans by the V α 24 and V β 11 rearrangement, and of Th2 cells, defined as CD294 (CRTH2)⁺CD3⁺ cells was determined by flow cytometry (please see gating strategy in Fig. 4.5), among sorted T-LPLs from normal intestinal mucosa of three control subjects and from inflamed intestinal mucosa of three clinically active patients with CD and three clinically active patients with UC (Fig. 4.6). The percentage of iNKT did not differ statistically between control, CD and UC T-LPLs (mean $0.23 \pm 0.18\%$; mean $0.34 \pm 0.10\%$; mean $0.05 \pm 0.04\%$, respectively). Finally, the mean percentage of Th2 cells was comparable between control, CD and UC T-LPLs ($1.43 \pm 0.50\%$; $1.14 \pm 0.20\%$;

$0.80 \pm 0.14\%$, respectively). The mean percentage of $V\alpha24^+V\beta11^-$ T cells which we observed were the following: control T-LPLs: $7.32 \pm 2.13\%$; CD T-LPLs: $22.31 \pm 22.64\%$; UC T-LPLs: $2.92 \pm 1.10\%$.

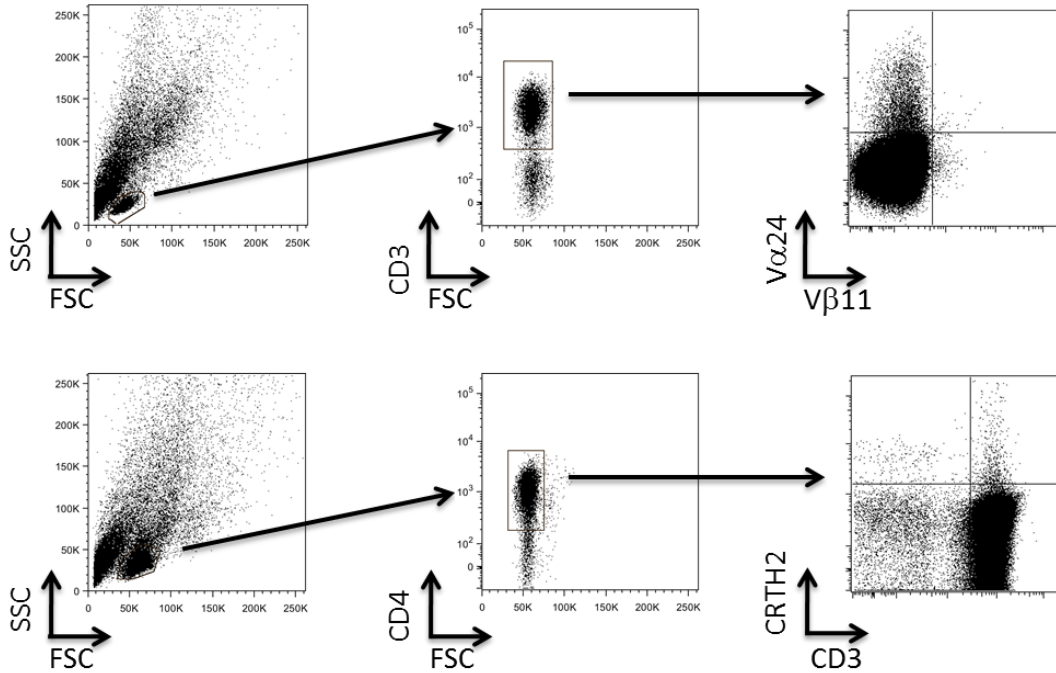


Figure 4.5

Figure 4.5. Gating strategy used for the evaluation of iNKT and Th2 cell distribution in the intestinal lamina propria. LPMCs were isolated from the intestinal mucosa of IBD patients and control subjects, and were stained with FITC-anti-human CD3, APC-anti-human CD4, PE-anti-human CD294 (CRTH2), PE-anti-human $V\alpha24$, APC -anti-human $V\beta11$. Subsequently, cells were analysed by flow cytometry. iNKT cells (**upper panels**) were identified as $V\alpha24^+V\beta11^+$ cells (gated on viable $CD3^+$ cells), whereas Th2 cells (**lower panels**) were identified as $CD294$ (CRTH2) $^+CD3^+$ cells (gated on viable $CD4^+$ cells). The dot plots display one experiment, and are representative of experiments performed in all patients with IBD and control subjects.

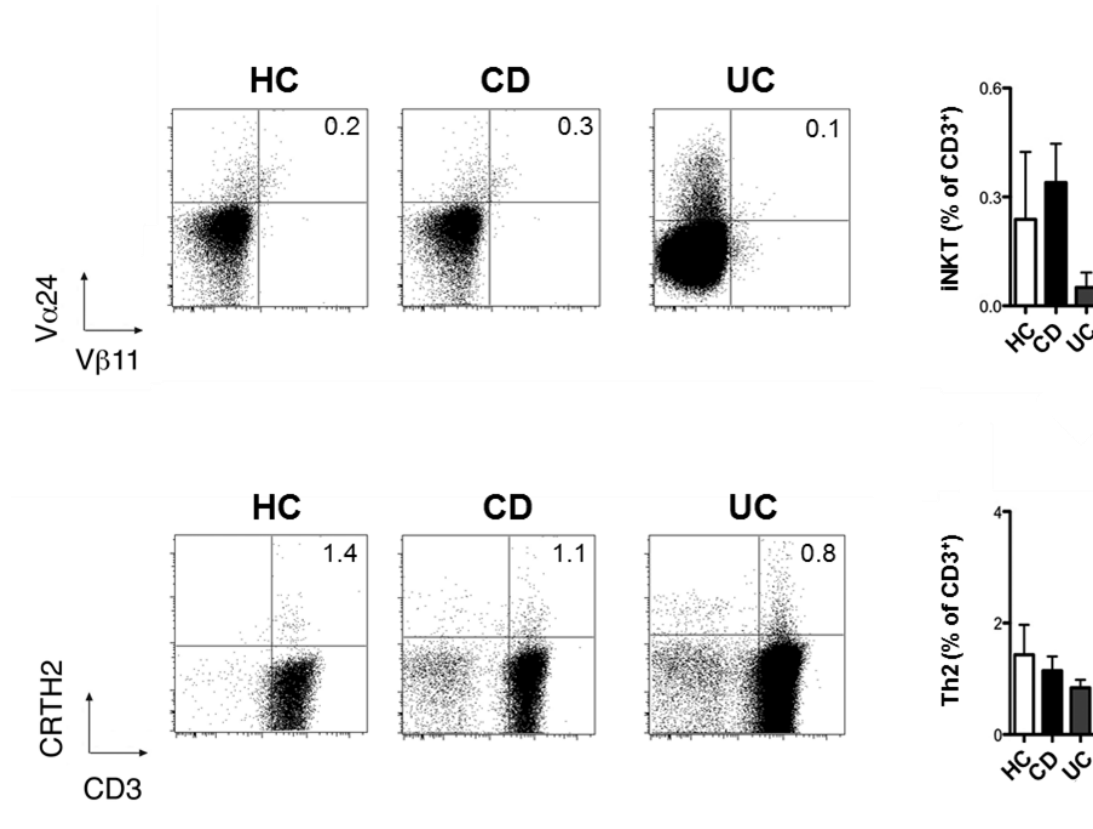


Figure 4.6

Figure 4.6. iNKT and Th2 cell distribution in the intestinal lamina propria. Relative distribution of iNKT cells (**upper panels**) and Th2 cells (**lower panels**) in T-LPLs of control subjects (HC, n=3) and patients with CD (n=3) and UC (n=3). Each dot plot displays one experiment, and is representative of experiments performed in all patients with IBD and control subjects. Right histogram bar graphs: mean percentage of iNKT cells (**upper panel**) or Th2 cells (**lower panel**) out of total viable T-LPLs from HC subjects, CD and UC patients. Bars represent mean \pm SEM. The Mann-Whitney *U* test was applied.

4.3.4 Percentage of IL-13-producing NKT cells after anti-CD3/CD28 stimulation

To determine the percentage of mucosal IL-13-producing cells in NKT cells after T cell activation, LPMCs from normal intestinal mucosa of three control subjects and from inflamed intestinal mucosa of four clinically active patients with CD and seven clinically active patients with UC were cultured in the presence of anti-

CD3/CD28 antibodies, subsequently evaluating the production of IL-13 by intracellular flow cytometry (please see gating strategy in Fig. 4.7). Basal IL-13 production by mucosal NKT cells was undetectable (data not shown). The percentage of IL-13⁺ NKT cells did not differ between control, CD and UC T-LPLs (mean $1.90 \pm 0.81\%$; mean $3.19 \pm 0.97\%$; mean $3.74 \pm 1.71\%$, respectively) (Fig. 4.8).

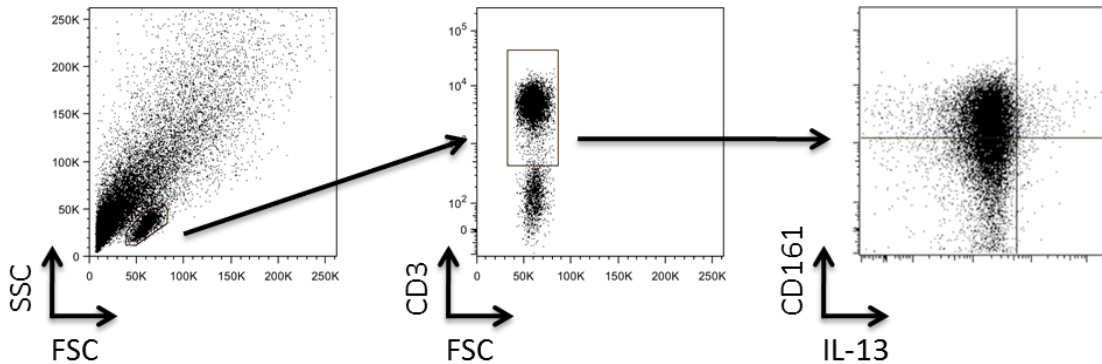


Figure 4.7

Figure 4.7. Gating strategy used for the evaluation of IL-13-producing NKT cells in the intestinal lamina propria. LPMCs were isolated from the intestinal mucosa of IBD patients and control subjects, and were stimulated for 48 hours with immobilised anti-CD3/CD28 monoclonal antibodies, with the addition of brefeldin A for the last 4 hours of culture. Intracellular IL-13 was measured on NKT cells. In particular, LPMCs were stained with FITC-anti-human CD3, APC-anti-human CD161 and, after permeabilisation, with PE-anti-human IL-13. Subsequently, cells were analysed by flow cytometry. NKT cells were identified as CD161⁺ (gated on viable CD3⁺ cells). The dot plots display one experiment, and are representative of experiments performed in all patients with IBD and control subjects.

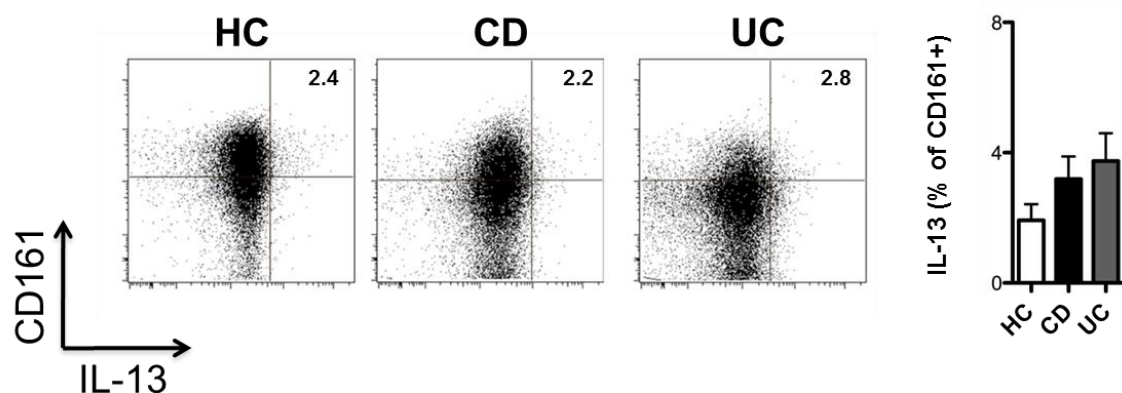


Figure 4.8

Figure 4.8. IL-13 production by NKT cells in the intestinal lamina propria. LPMCs isolated from intestinal mucosa of control subjects (HC, n=3) and from inflamed intestinal mucosa of clinically active patients with CD (n=4) and UC (n=7) were stimulated for 48 hours with immobilised anti-CD3/CD28 monoclonal antibodies, and intracellular IL-13 was subsequently measured on NKT cells. Each dot plot displays one experiment, and is representative of experiments performed in all patients with IBD and control subjects. Right histogram bar graph: mean percentage of IL-13-producing NKT cells out of total viable T-LPLs from HC subjects and CD and UC patients. Data are shown as mean \pm SEM. The Mann-Whitney *U* test was applied.

4.3.5 *In vitro* production of IL-13, IFN- γ , IL-17A, IL-4 and IL-5 by LPMCs after activation with anti-CD3/CD28 antibodies

The production of IL-13, IL-17A, IFN- γ , IL-4 and IL-5 by unstimulated and anti-CD3/CD28-stimulated LPMCs from the inflamed mucosa of 22 IBD patients (11 with CD and 11 with UC) and the normal mucosa of 11 control subjects was then evaluated by ELISA (Fig. 4.9, Fig. 4.10 and Fig. 4.11).

No significant difference was found between control, CD and UC LPMCs in the production of IL-13 in unstimulated conditions (control subjects: median 12 pg/ml, range 0-40; CD: median 27 pg/ml, range 0-78; UC: median 30 pg/ml, range 0-72) (Fig. 4.9). In unstimulated conditions, LPMCs from CD and UC patients produced significantly ($p < 0.05$) higher amounts of IFN- γ (median 547 pg/ml, range 170-

832; UC: median 373 pg/ml, range 210-902) in comparison with control LPMCs (median 134 pg/ml, range 27-321). In unstimulated conditions, LPMCs from CD and UC patients produced significantly ($p<0.05$) higher amounts of IL-17A (median 392 pg/ml, range 120-1330; UC: median 436 pg/ml, range 138-532) in comparison with control LPMCs (median 59 pg/ml, range 19-242). No difference in IFN- γ and IL-17A production was found between unstimulated CD and UC LPMCs (Fig. 4.9).

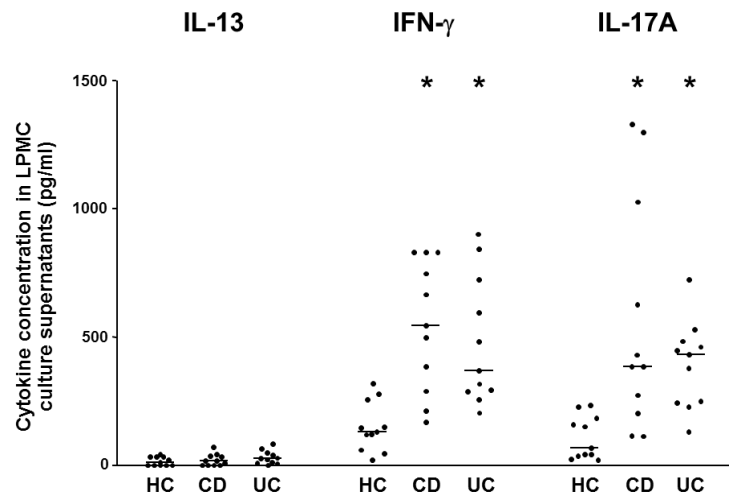


Figure 4.9

Figure 4.9. *In vitro* production of IL-13, IFN- γ and IL-17A by unstimulated LPMCs. Levels of IL-13, IFN- γ and IL-17A, expressed in pg/ml, in the supernatants of unstimulated LPMCs isolated from the inflamed intestinal mucosa of 11 CD patients and 11 UC patients, and from the mucosa of 11 control subjects (HC), and cultured for 48 hours. Horizontal bars are median values. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test. * $p<0.05$ versus concentration of the same cytokine in culture supernatants from HC LPMCs.

Stimulation with anti-CD3/CD28 antibodies induced a significant ($p<0.05$) increase in IL-13 production by both CD and UC LPMCs, but did not influence significantly the release of IL-13 by control LPMCs (control subjects: median 40 pg/ml, range 0-141; CD: median 38 pg/ml, range 0-190; UC: median 63 pg/ml, range 0-143) (Fig. 4.10). No significant difference in IL-13 production by anti-CD3/CD28-stimulated LPMCs was found between control, CD and UC LPMCs. Of note, IL-13 levels in culture supernatants were never greater than 200 pg/ml.

After stimulation with anti-CD3/CD28 antibodies, IFN- γ production was significantly ($p < 0.0005$) enhanced in comparison with unstimulated conditions in CD, UC and control subjects (CD: median 7281 pg/ml, range 4351-12210; UC: median 5432 pg/ml, range 2203-12302; control subjects: median 2026 pg/ml, range 951-6149). Of note, in three out of 11 CD and two out of 11 UC patients IFN- γ production exceeded 10000 pg/ml. Activation with anti-CD3/CD28 antibodies also significantly enhanced also the production of IL-17A in CD, UC and control subjects (CD: median 6332 pg/ml, range 2079-12900, $p < 0.0005$; UC: median 4232 pg/ml, range 2018-11200, $p < 0.0005$; control subjects: median 1730 pg/ml, range 830-5555, $p < 0.005$). In three out of 11 CD and two out of 11 UC patients IL-17A concentration exceeded 10000 pg/ml. Anti-CD3/CD28 stimulation markedly increased IFN- γ and IL-17A release by LPMCs from all 11 CD, 11 UC patients and 11 control subjects. Conversely, the overall increase in IL-13 production observed in CD and UC LPMCs upon anti-CD3/CD28 stimulation was mostly due to a subset of six out of 11 CD patients and five out of 11 UC patients who responded to anti-CD3/CD28 stimulation. Culture with anti-CD3/CD28 antibodies enhanced IL-13 production by LPMCs from only four out of 11 control subjects.

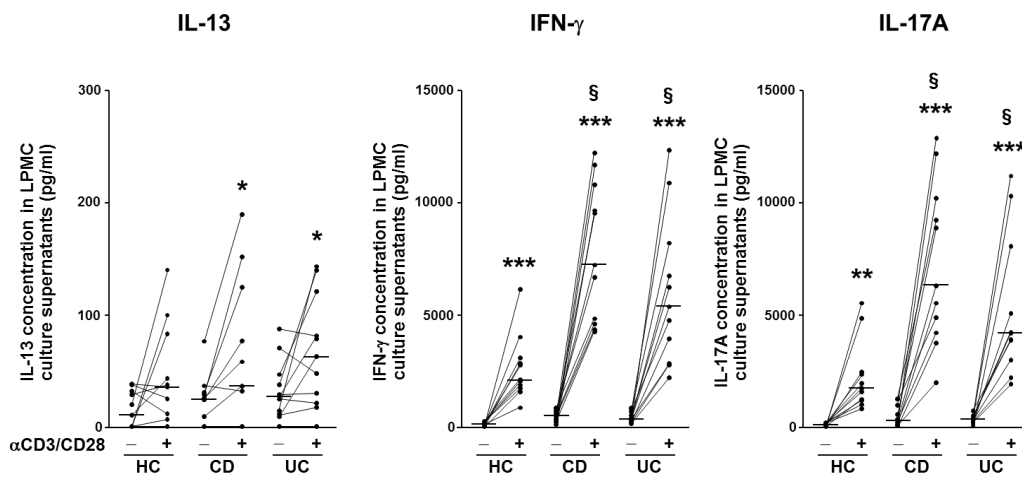


Figure 4.10

Figure 4.10. *In vitro* production of IL-13, IFN- γ and IL-17A by activated LPMCs. Levels of IL-13, IFN- γ and IL-17A, expressed in pg/ml, in the supernatants of unstimulated (-) and anti- (α)CD3/CD28-stimulated (+) LPMCs isolated from the inflamed intestinal mucosa of 11 CD patients and 11 UC

patients, and from the mucosa of 11 control subjects (HC), and cultured for 48 hours. Each set of connected points represents data from one patient. Horizontal bars are median values. The paired two-tailed *t* test was applied to compare between unstimulated and stimulated conditions, and the ANOVA One way analysis of variance followed by the Tukey's multiple comparison test was applied to compare the same condition of culture in different patient populations.

* $p < 0.05$ versus unstimulated LPMCs of the respective group of patients;
 ** $p < 0.005$ versus unstimulated LPMCs of the respective group of patients;
 *** $p < 0.0005$ versus unstimulated LPMCs of the respective group of patients;
 § $p < 0.05$ versus α CD3/CD28-stimulated HC LPMCs.

IL-4 and IL-5 were undetectable in all the supernatants of unstimulated or anti-CD3/CD28-stimulated LPMCs from both IBD and control mucosa (Fig. 4.11).

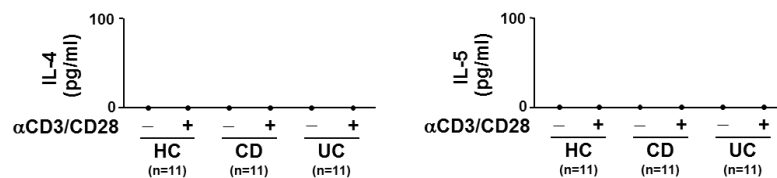


Figure 4.11

Figure 4.11. *In vitro* production of IL-4 and IL-5 by LPMCs. Levels of IL-4 and IL-5, expressed in pg/ml, in the supernatants of unstimulated (-) and anti-(α)CD3/CD28-stimulated (+) LPMCs isolated from the inflamed intestinal mucosa of 11 CD patients and 11 UC patients, and from the mucosa of 11 control subjects (HC), and cultured for 48 hours. Each set of connected points represents data from one patient. Horizontal bars are median values. The paired two-tailed *t* test was applied to compare between unstimulated and stimulated conditions, and the ANOVA One way analysis of variance followed by the Tukey's multiple comparison test was applied to compare the same condition of culture in different patient populations.

4.3.6 *In vitro* production of IL-13, IFN- γ , IL-17A, IL-4 and IL-5 by LPMCs after activation with anti-CD2/CD28 antibodies

The production of IL-13, IL-17A, IFN- γ , IL-4 and IL-5 by anti-CD2/CD28-stimulated LPMCs isolated from the inflamed mucosa of eight different IBD

patients (four with CD and four with UC) and the normal mucosa of four different control subjects was also investigated (Table 4.5). No significant difference was found in IL-13 production between control, CD and UC LPMCs in unstimulated and anti-CD2/CD28-stimulated conditions. In contrast, anti-CD2/CD28 stimulation significantly enhanced IFN- γ and IL-17A production by CD and UC LPMCs, although IFN- γ and IL-17A in culture supernatants of anti-CD2/28-stimulated LPMCs was 2-3 fold lower than in supernatants of anti-CD3/CD28-stimulated LPMCs. Anti-CD2/CD28 stimulation led to a marked increase in IFN- γ and IL-17A production by control LPMCs, however this was not statistically significant after applying the paired two-tailed *t* test. IL-4 and IL-5 were undetectable in all the supernatants of unstimulated or anti-CD2/CD28-stimulated LPMCs from both IBD and control mucosa.

Table 4.5. IL-13, IFN- γ , IL-17A, IL-4 and IL-5 production by anti-(α)CD2/CD28-stimulated LPMCs. IL-13, IFN- γ , IL-17A, IL-4 and IL-5 levels (pg/ml) in the supernatants of unstimulated and α CD2/CD28-stimulated LPMCs isolated from the inflamed intestinal mucosa of four CD and four UC patients, and from the intestinal mucosa of four control subjects (HC), and cultured for 48 hours. Results are shown as mean \pm SEM. The paired two-tailed *t* test was applied to compare between unstimulated and stimulated conditions, and the ANOVA One way analysis of variance followed by the Tukey's multiple comparison test was applied to compare the same condition of culture in different patient populations. **p*<0.05 *versus* unstimulated LPMCs of the respective group of patients; \S *p*<0.05 *versus* α CD3/CD28-stimulated HC LPMCs.

	HC		CD		UC	
	Medium	α CD2/CD28	Medium	α CD2/CD28	Medium	α CD2/CD28
IL-13	18 \pm 3	18 \pm 5	27 \pm 5	30 \pm 4	27 \pm 5	31 \pm 6
IFN-γ	133 \pm 17	994 \pm 270	512 \pm 144	4918 \pm 707* \S	478 \pm 78	3336 \pm 801* \S
IL-17A	70 \pm 28	486 \pm 139	327 \pm 39	3453 \pm 591* \S	490 \pm 60	3065 \pm 614* \S
IL-4	0	0	0	0	0	0
IL-5	0	0	0	0	0	0

4.3.7 *In vivo* expression of IL-13 and IL-5 transcripts in intestinal mucosa

We thought it important to use another methodology to analyse IL-13, IL-5, IFN- γ and IL-17A in IBD mucosa so we measured cytokine transcripts by qRT-PCR in snap frozen fresh intestinal biopsies from inflamed mucosa of seven patients with UC, nine patients with CD and from normal mucosa of 16 control subjects (Fig. 4.12). The mean expression of IL-13 was uniformly low in all groups studied, and IL-5 expression was not significantly different in UC, CD and control mucosa. IFN- γ transcripts were significantly higher in CD patients in comparison with control subjects. As previously described (Fujino *et al.* 2003), IL-17A transcripts were also up-regulated in CD mucosa.

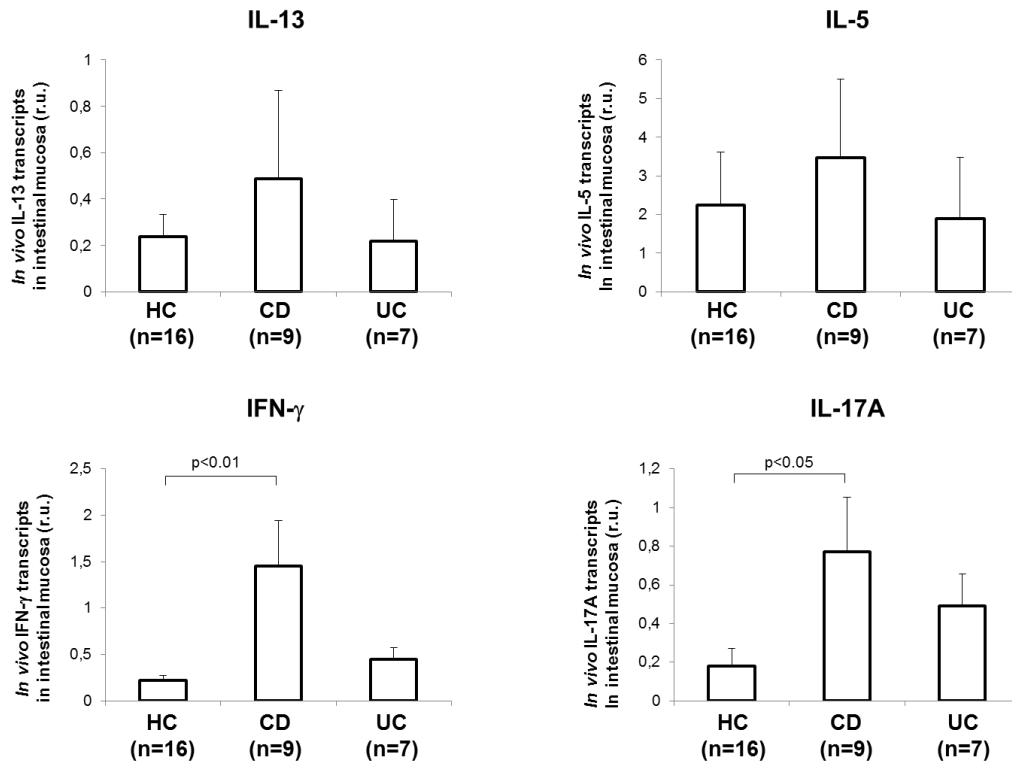


Figure 4.12

Figure 4.12. *In vivo* mucosal expression of IL-13 and IL-5. Expression of IL-13, IL-5, IFN- γ and IL-17A in inflamed intestinal mucosa of seven patients with UC, nine patients with CD, and from normal mucosa of 16 control subjects (HC). Levels were normalised to the mean expression of 18S, β -actin and GAPDH. Data are shown as mean \pm SEM of the indicated number of donors, each examined once. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test. r.u., relative units.

4.3.8 *Ex vivo* production of IL-13, IL-1 β , collagen and TGF- β 1 in strictured CD tissue

Intestinal muscle layer explants (1mm³ in size) were then cultured *ex vivo* for 24 hours from uninflamed areas of strictured and non-strictured ileum of 6 patients with fibrostenosing CD and from normal ileum of 7 control subjects, and IL-13, IL-1 β and collagen concentration in the culture supernatant and TGF- β 1 transcripts in the cultured tissue were measured (Fig. 4.13). The concentrations of IL-13 were uniformly low in all samples (strictured CD ileum: median 24 pg/ml, range 15-30; non-strictured CD ileum: median 19 pg/ml, range 9-28; normal ileum of control subjects: median 22 pg/ml, range 16-35).

In contrast, both IL-1 β and collagen were significantly higher in the organ culture supernatants of strictured CD ileum (median 372 pg/ml, range 226-595; median 1159 μ g/ml, range 603-1429, respectively) compared with those of non-strictured CD ileum (median 188 pg/ml, range 95-255, $p < 0.01$; median 508 μ g/ml, range 248-958, $p < 0.05$, respectively) and normal ileum of control subjects (median 111 pg/ml, range 52-320, $p < 0.01$; median 495 μ g/ml, range 325-933; $p < 0.01$, respectively). Similarly, TGF- β 1 mRNA was significantly ($p < 0.001$) higher in the cultured muscle layer tissue from strictured CD ileum compared with that in non-strictured CD ileum and normal ileum of control subjects.

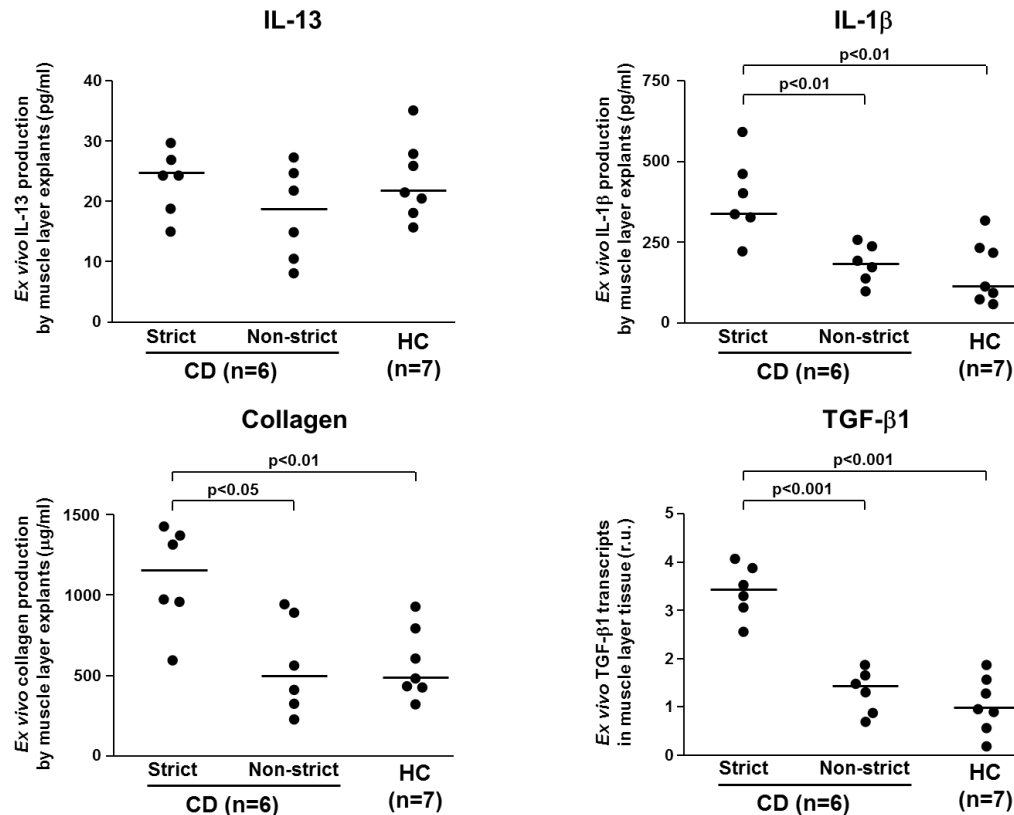


Figure 4.13

Figure 4.13. Ex vivo production of IL-13 by strictured CD tissue. Levels of IL-13 and IL-1 β , expressed as pg/ml, and collagen, expressed as μ g/ml, in the supernatants of muscle layer explants from uninflamed areas of strictured (Strict) and non-strictured (Non-strict) ileum of 6 patients with fibrostenosing CD and from normal ileum of 7 control subjects (HC), cultured for 24 hours in the absence of stimuli, and levels of TGF- β 1 mRNA, expressed as relative units compared with the median expression in HCs that was assigned the value 1 in the same cultured tissue explants. Horizontal bars are median values. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test. r.u., relative units.

4.3.9 Surface expression of IL-4R α , IL-13R α 1 and IL-13R α 2 on intestinal T cells and macrophages

In parallel to the evaluation of IL-13 expression in normal and inflamed intestinal mucosa, we aimed to characterise which cell types may respond to IL-13 in the intestinal lamina propria of IBD patients and control subjects. In order to do so,

we used flow cytometry to measure IL-4R α , IL-13R α 1 and IL-13R α 2 expression on the surface of both CD3⁺ and CD68⁺ LPMCs (please see gating strategy in Fig. 4.14) from three control subjects, from inflamed colonic mucosa of three UC patients, from inflamed colonic mucosa of three CD patients and from uninflamed ileal mucosa overlying strictured and non-strictured areas of three CD patients (Fig. 4.15, Fig. 4.16). Surface expression of IL-4 α , IL-13R α 1 and IL-13R α 2 by LPMCs isolated from normal mucosa of control subjects, from inflamed UC and CD mucosa and from the mucosa overlying CD strictures and CD mucosa above non-strictured areas was very low or not detectable, with no significant difference between all the groups of patients studied, in both T cells (Fig. 4.15) and macrophages (Fig. 4.16).

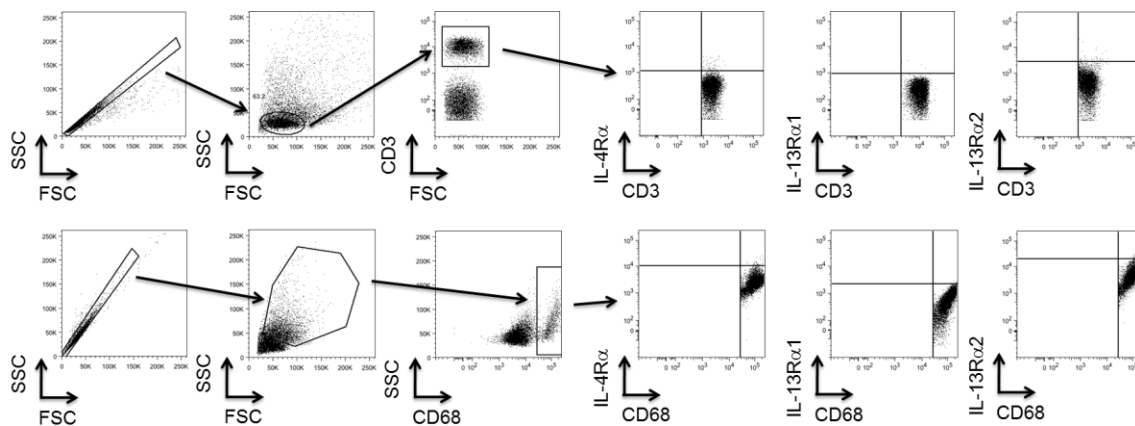


Figure 4.14

Figure 4.14. Gating strategy used for the evaluation of surface expression of IL-4R α , IL-13R α 1 and IL-13R α 2 on intestinal T cells and macrophages. LPMCs were isolated from the intestinal mucosa of IBD patients and control subjects, and were stained with FITC-anti-human CD3, APC-anti-human IL-4R α , Alexa Fluor[®] 700-anti-human IL-13R α 1, fluorescein-anti-human IL-13R α 2, and, after permeabilisation, with biotinylated anti-human CD68. Subsequently, cells were analysed by flow cytometry. The doublets corresponding to cell aggregates were excluded by gating, and single cells were further analysed. Events were also gated to exclude nonviable cells on the basis of light scatter, and to eliminate artefacts caused by poor flow. T cells (**upper panels**) were identified as CD3⁺ cells, whereas macrophages (**lower panels**) were identified as CD68⁺ cells. The dot plots display one experiment, and are representative of experiments performed in all IBD patients and control subjects.

Figure 4.15 (see next page). **Surface expression of IL-4R α , IL-13R α 1 and IL-13R α 2 on intestinal T cells.** The expression of IL-4R α , IL-13R α 1 and IL-13R α 2 on the surface of CD3-gated LPMCs from the normal colon of a control subject (HC), from inflamed colonic mucosa of a UC patient, from inflamed colonic mucosa of a CD patient, and from uninfamed ileal mucosa overlying strictured and non-strictured areas of a fibrostenosing CD patient was determined by flow cytometry. Dashed line histograms display isotype control, while solid line histograms correspond to the receptor expression. Each histogram corresponds to one representative sample per group of independent experimental repeats (one for each patient analysed). The example is representative of independent experiments performed in three HC subjects, three UC patients, three luminal CD patients and three fibrostenosing CD patients. Data were analysed by ANOVA. One way analysis of variance followed by the Tukey's multiple comparison test.

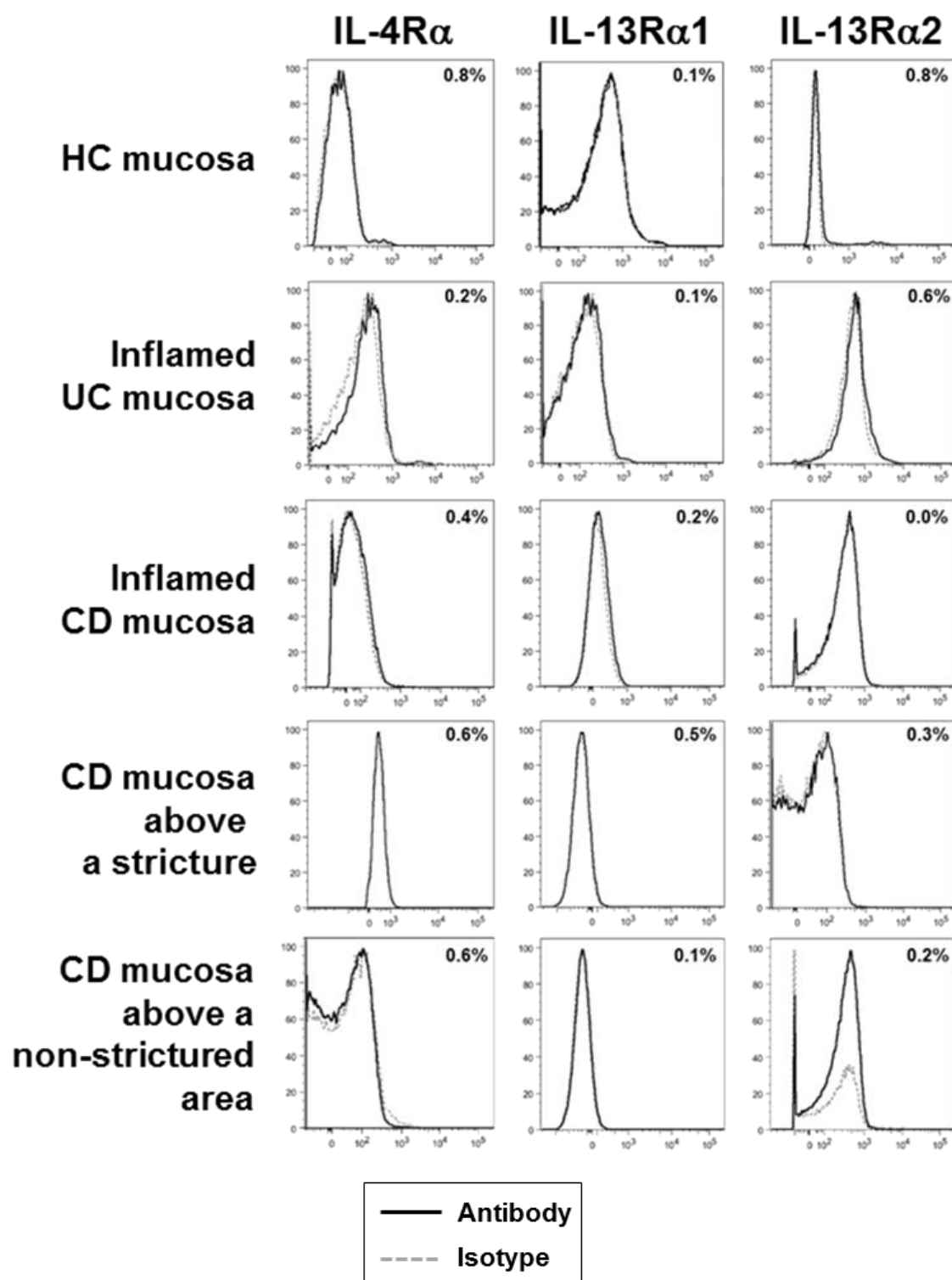


Figure 4.15

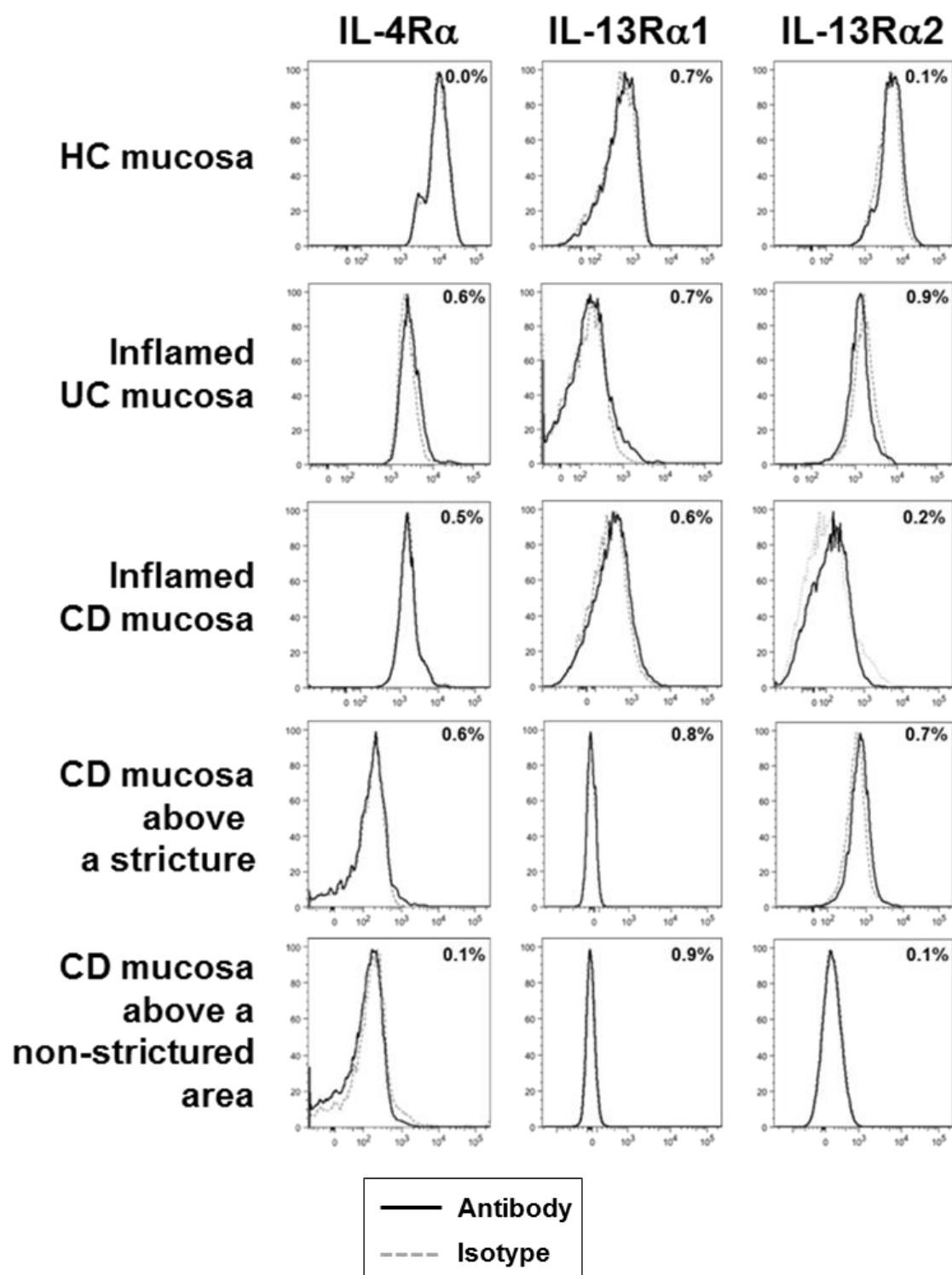


Figure 4.16

Figure 4.16 (see previous page). **Surface expression of IL-4R α , IL-13R α 1 and IL-13R α 2 on intestinal macrophages.** The expression of IL-4R α , IL-13R α 1 and IL-13R α 2 on the surface of CD68-gated LPMCs from the normal colon of a control subject (HC), from inflamed colonic mucosa of a UC patient, from inflamed colonic mucosa of a CD patient, and from uninflamed ileal mucosa overlying strictured and non-strictured areas of a fibrostenosing CD patient was determined by flow cytometry. Dashed line histograms display isotype control, while solid line histograms correspond to the receptor expression. Each histogram corresponds to one representative sample per group of independent experimental repeats (one for each patient analysed). The example is representative of independent experiments performed in three HC subjects, three UC patients, three luminal CD patients and three fibrostenosing CD patients. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test.

4.3.10 Cytoplasmic expression of IL-4R α , IL-13R α 1 and IL-13R α 2 in intestinal T cells

Alongside the detection of IL-13R expression on the cell surface, we used part of the same LPMCs from IBD patients and control subjects for the assessment of intracellular expression of IL-4R α , IL-13R α 1 and IL-13R α 2. In particular, we assessed by flow cytometry IL-4R α , IL-13R α 1 and IL-13R α 2 expression in the cytoplasm of CD3⁺ LPMCs (please see gating strategy in Fig. 4.17) from three control subjects, from inflamed colonic mucosa of three UC patients, from inflamed colonic mucosa of three CD patients and from uninflamed ileal mucosa overlying strictured and non-strictured areas of three CD patients (Fig. 4.18). The intracellular expression of IL-4R α and IL-13R α 2 by CD3⁺ LPMCs isolated from normal mucosa of control subjects, from inflamed UC and CD mucosa and from the mucosa overlying CD strictures and CD mucosa above non-strictured areas was very low or not detectable. Conversely, CD3⁺ LPMCs from inflamed CD mucosa expressed significantly ($p < 0.001$) higher intracellular levels of IL-13R α 1 (mean $33.5 \pm 12.5\%$) than CD3⁺ LPMCs from normal mucosa of control subjects (mean $1.2 \pm 0.3\%$), from inflamed UC mucosa (mean $1.4 \pm 0.7\%$) and from the mucosa overlying CD strictures (mean $0.8 \pm 0.3\%$) and CD mucosa above non-

stricted areas (mean $1.1 \pm 0.6\%$).

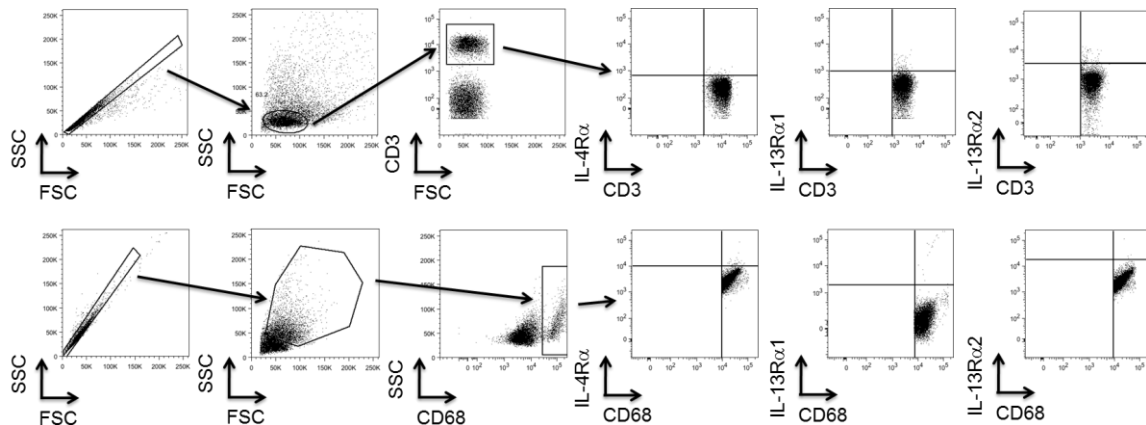


Figure 4.17

Figure 4.17. Gating strategy used for the evaluation of intracellular expression of IL-4R α , IL-13R α 1 and IL-13R α 2 in intestinal T cells and macrophages. LPMCs were isolated from the intestinal mucosa of IBD patients and control subjects, and were stained with FITC-anti-human CD3 and, after permeabilisation, with APC-anti-human IL-4R α , Alexa Fluor[®] 700-anti-human IL-13R α 1, fluorescein-anti-human IL-13R α 2 and biotinylated anti-human CD68. Subsequently, cells were analysed by flow cytometry. The doublets corresponding to cell aggregates were excluded by gating, and single cells were further analysed. Events were also gated to exclude nonviable cells on the basis of light scatter, and to eliminate artefacts caused by poor flow. T cells (**upper panels**) were identified as CD3⁺ cells, whereas macrophages (**lower panels**) were identified as CD68⁺ cells. The dot plots display one experiment, and are representative of experiments performed in all IBD patients and control subjects.

Figure 4.18 (see next page). **Intracellular expression of IL-4R α , IL-13R α 1 and IL-13R α 2 in intestinal T cells.** The intracellular expression of IL-4R α , IL-13R α 1 and IL-13R α 2 by CD3-gated LPMCs from the normal colon of a control subject (HC), from inflamed colonic mucosa of a UC patient, from inflamed colonic mucosa of a CD patient, and from uninflamed ileal mucosa overlying strictured and non-strictured areas of a fibrostenosing CD patient was determined by flow cytometry. Dashed line histograms display isotype control, while solid line histograms correspond to the receptor expression. Each histogram corresponds to one representative sample per group of independent experimental repeats (one for each patient analysed). The example is representative of independent experiments performed in three HC subjects, three UC patients, three luminal CD patients and three fibrostenosing CD patients. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test.

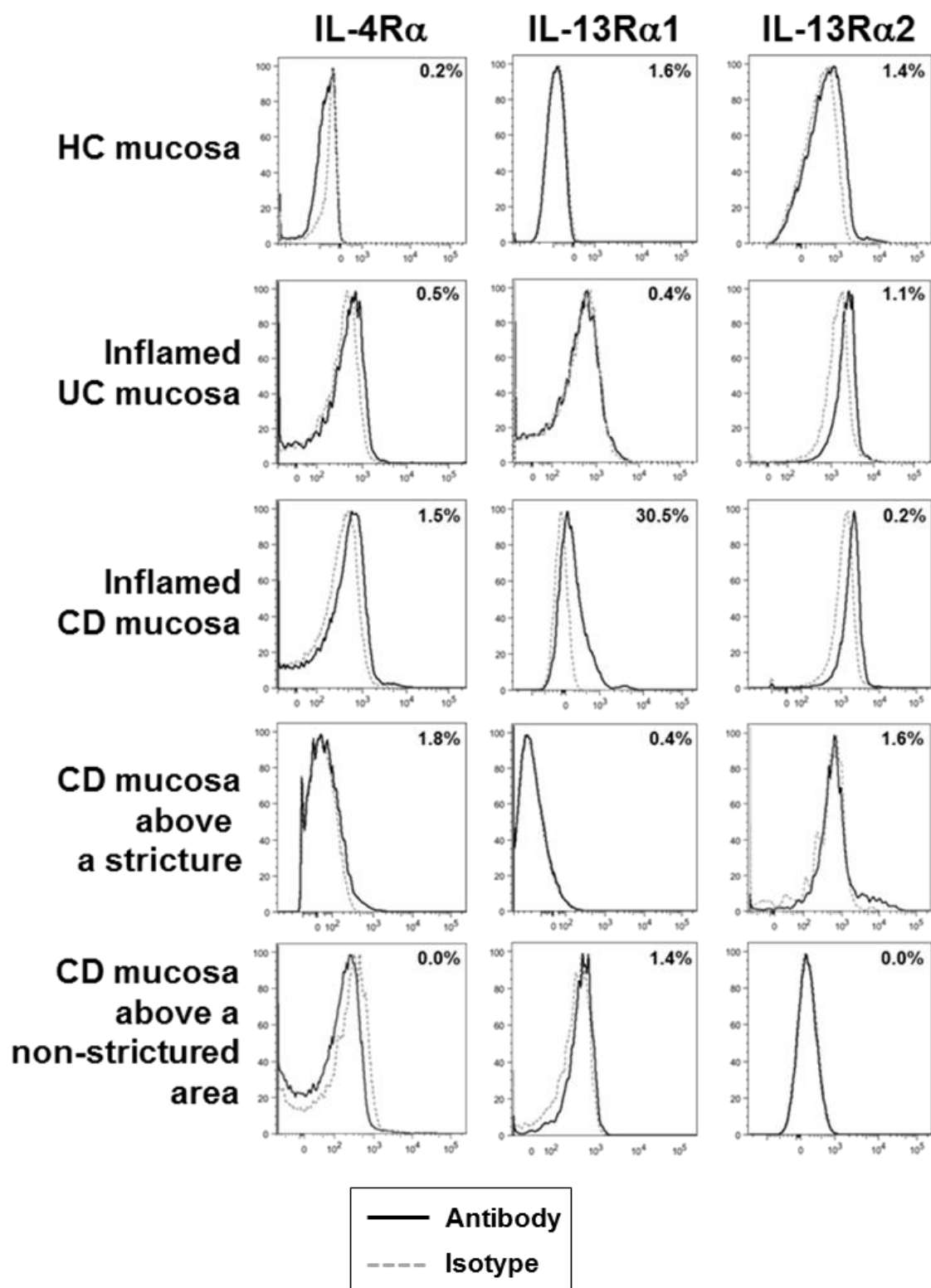


Figure 4.18

4.3.11 Cytoplasmic expression of IL-4R α , IL-13R α 1 and IL-13R α 2 in intestinal macrophages

In parallel, on the same LPMCs used for assessing intracellular IL-13R expression in T cells, we assessed by flow cytometry intracellular expression of IL-13Rs in CD68⁺ cells. In particular, we assessed by flow cytometry IL-4R α , IL-13R α 1 and IL-13R α 2 expression in the cytoplasm of CD68⁺ LPMCs (please see gating strategy in Fig. 4.17) from three control subjects, from inflamed colonic mucosa of three UC patients, from inflamed colonic mucosa of three CD patients and from uninflamed ileal mucosa overlying strictured and non-strictured areas of three CD patients (Fig. 4.19). The intracellular expression of IL-4R α and IL-13R α 2 by CD68⁺ LPMCs isolated from normal mucosa of control subjects, from inflamed UC and CD mucosa and from the mucosa overlying CD strictures and CD mucosa above non-strictured areas was very low or not detectable. Conversely, CD68⁺ LPMCs from inflamed CD mucosa expressed significantly ($p < 0.001$) higher intracellular levels of IL-13R α 1 (mean $52.3 \pm 21.7\%$) than CD68⁺ LPMCs from normal mucosa of control subjects (mean $0.9 \pm 0.6\%$), from inflamed UC mucosa (mean $1.6 \pm 0.4\%$) and from the mucosa overlying CD strictures (mean $0.7 \pm 0.5\%$) and CD mucosa above non-strictured areas (mean $0.9 \pm 0.3\%$).

Figure 4.19 (see next page). **Intracellular expression of IL-4R α , IL-13R α 1 and IL-13R α 2 in intestinal macrophages.** The intracellular expression of IL-4R α , IL-13R α 1 and IL-13R α 2 by CD68-gated LPMCs from the normal colon of a control subject (HC), from inflamed colonic mucosa of a UC patient, from inflamed colonic mucosa of a CD patient, and from uninflamed ileal mucosa overlying strictured and non-strictured areas of a fibrostenosing CD patient was determined by flow cytometry. Dashed line histograms display isotype control, while solid line histograms correspond to the receptor expression. Each histogram corresponds to one representative sample per group of independent experimental repeats (one for each patient analysed). The example is representative of independent experiments performed in three HC subjects, three UC patients, three luminal CD patients and three fibrostenosing CD patients. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test.

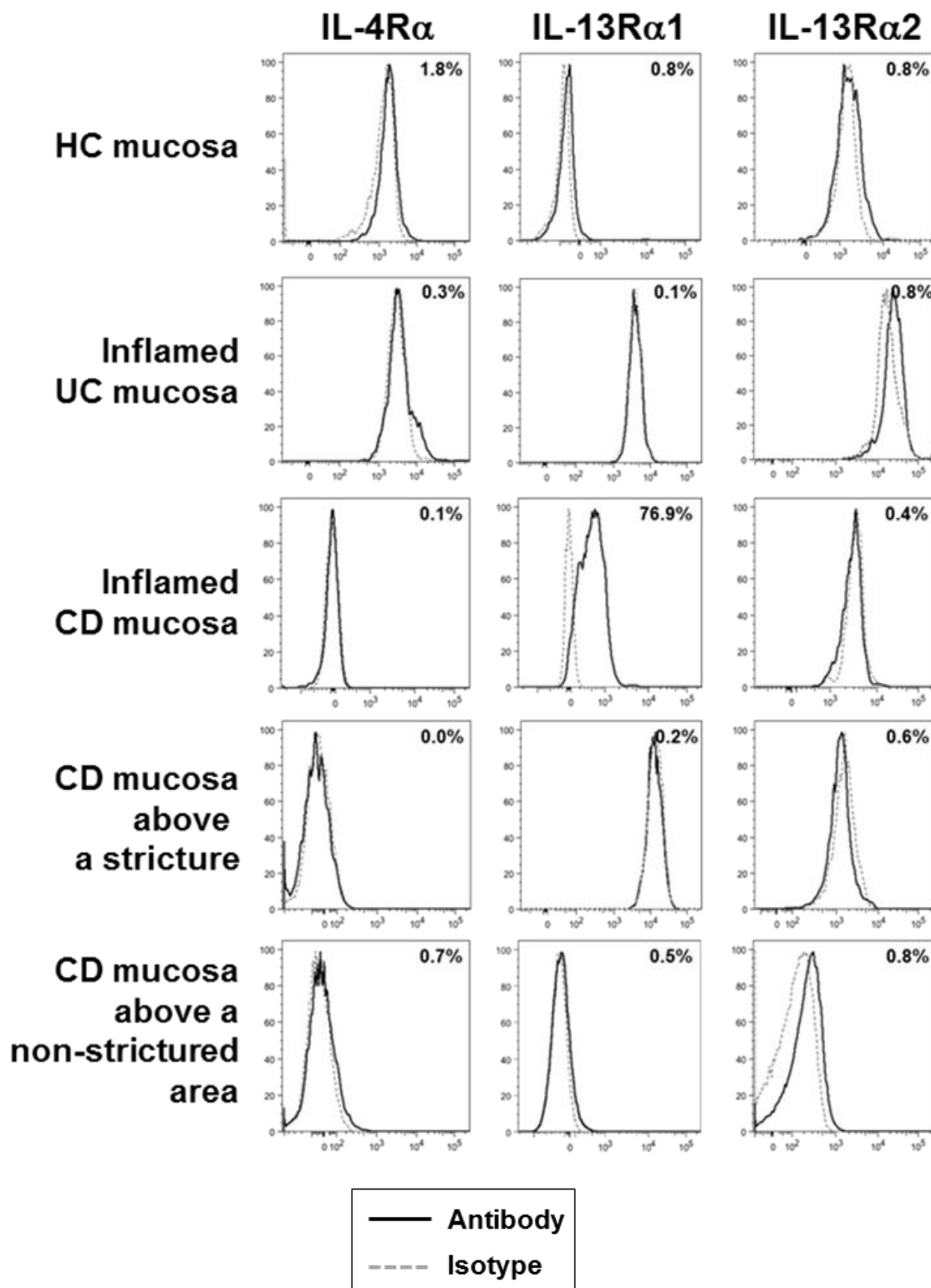


Figure 4.19

4.3.12 Expression of IL-4R α , IL-13R α 1 and IL-13R α 2 by intestinal myofibroblasts

We also investigated by flow cytometry the expression of IL-4R α , IL-13R α 1 and IL-13R α 2 on the surface and in the cytoplasm of submucosal myofibroblasts (please see gating strategy in Fig. 4.20) isolated from uninflamed areas of strictured and non-strictured ileum of three patients with fibrostenosing CD, and from normal ileum of three control subjects (Fig. 4.21). Surface expression of IL-4R α , IL-13R α 1 and IL-13R α 2 by myofibroblasts isolated from uninflamed areas of strictured and non-strictured CD ileum and from control ileum was very low or not detectable (Fig. 4.21A).

Myofibroblasts isolated from uninflamed areas of strictured CD ileum expressed significantly ($p < 0.05$) higher intracellular levels of IL-4R α (mean $6.2 \pm 2.7\%$) than myofibroblasts isolated from uninflamed areas of non-strictured CD ileum (mean $0.6 \pm 0.4\%$) and from control ileum (mean $0.4 \pm 0.3\%$) (Fig. 4.21B). Myofibroblasts isolated from uninflamed areas of both strictured and non-strictured CD ileum expressed significantly ($p < 0.05$) higher intracellular levels of IL-13R α 1 (mean $21.5 \pm 8.6\%$; mean $18.3 \pm 5.5\%$, respectively) and IL-13R α 2 (mean $61.2 \pm 21.1\%$; mean $52.6 \pm 16.2\%$, respectively) than myofibroblasts isolated from control ileum (IL-13R α 1: mean $6.9 \pm 2.8\%$; IL-13R α 2: mean $11.2 \pm 4.7\%$).

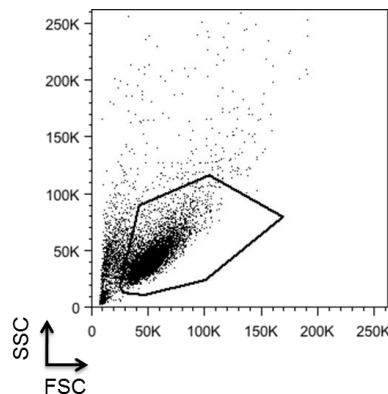


Figure 4.20

Figure 4.20 (see previous page). **Gating strategy used for the evaluation of IL-4R α , IL-13R α 1 and IL-13R α 2 expression by intestinal myofibroblasts.** Submucosal intestinal myofibroblasts were isolated from CD patients and control subjects, and were then stained, before or after permeabilisation, with APC-anti-human IL-4R α , Alexa Fluor[®] 700-anti-human IL-13R α 1 and fluorescein-anti-human IL-13R α 2. Subsequently, cells were analysed by flow cytometry. The dot plot displays one experiment, and is representative of experiments performed in all CD patients and control subjects.

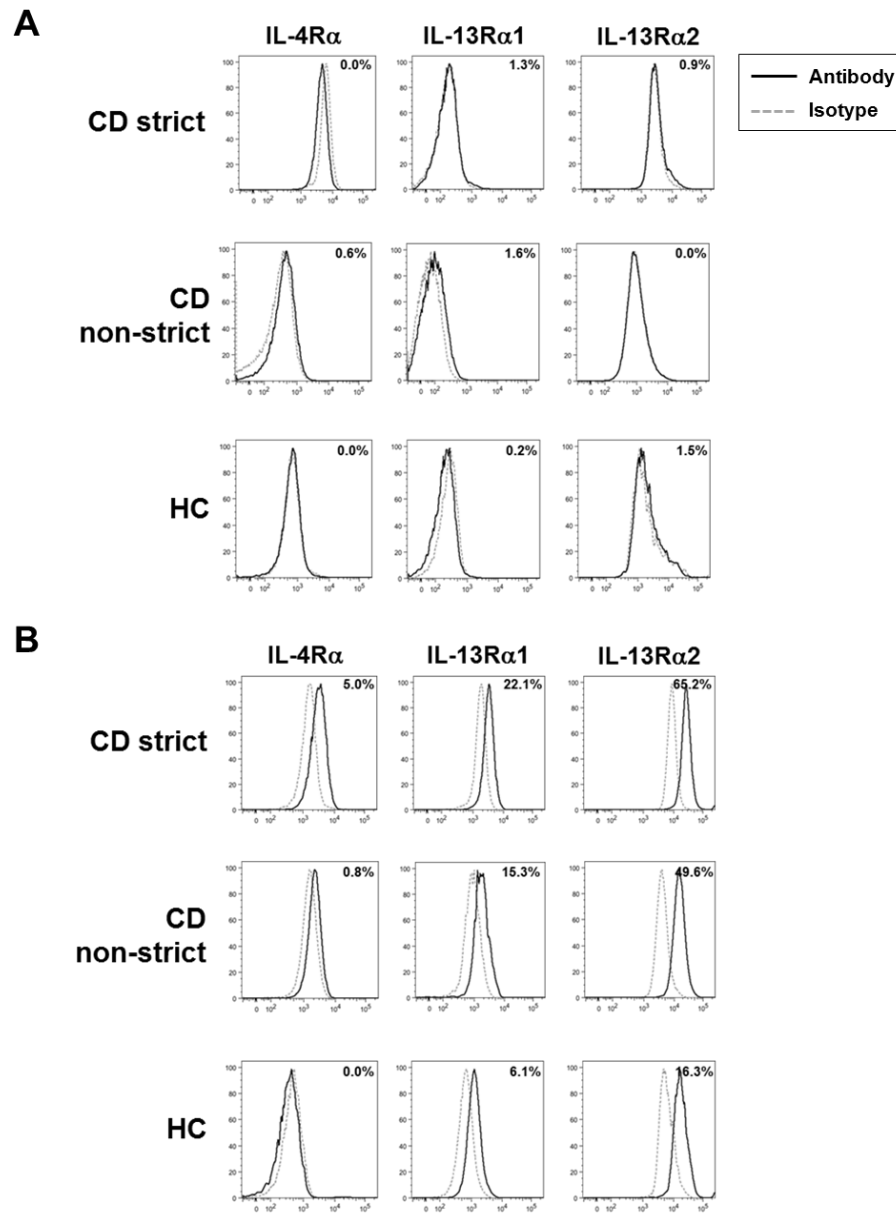


Figure 4.21

Figure 4.21 (see previous page). **IL-4R α , IL-13R α 1 and IL-13R α 2 expression by intestinal myofibroblasts.** (A) Surface or (B) intracellular expression of IL-4R α , IL-13R α 1 and IL-13R α 2 by submucosal myofibroblasts isolated from uninfamed areas of strictured and non-strictured ileum of a patient with fibrostenosing CD, and from normal ileum of a control subject (HC) was determined by flow cytometry. Dashed line histograms display isotype control, while solid line histograms correspond to the receptor expression. Each histogram corresponds to one representative sample per group of independent experimental repeats (one for each patient analysed). The example is representative of independent experiments performed in three fibrostenosing CD patients and three HC. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test.

4.3.13 Effect of IL-13R α 2 and IL-4R α neutralisation on IL-13 production in UC

Concentrations of cytokines in supernatants reflect both production and consumption. Since IL-13 can bind both to IL-13R α 2 and to the heterodimer formed by IL-13R α 1 and IL-4R α (Rahaman *et al.* 2002; LaPorte *et al.* 2008), LPMCs from inflamed UC with anti-CD3/CD28 or anti-CD2/CD28 antibodies were cultured in the absence or presence of anti-IL-13R α 2 and anti-IL-4R α neutralising antibodies or their control IgG, and IL-13 in the culture supernatants was measured by ELISA (Fig. 4.22). IL-13, IFN- γ and IL-17A concentrations in the supernatants of anti-CD3/CD28-stimulated UC LPMCs cultured with anti-IL-13R α 2 and anti-IL-4R α antibodies were not significantly different than those in the supernatants of anti-CD3/CD28-stimulated UC LPMCs cultured with the controls (Fig. 4.22). Similar results were observed in the supernatants of anti-CD2/CD28-stimulated LPMCs (data not shown). These experiments suggest that consumption of IL-13 by LPMCs is not the reason for the low levels I observed in IBD.

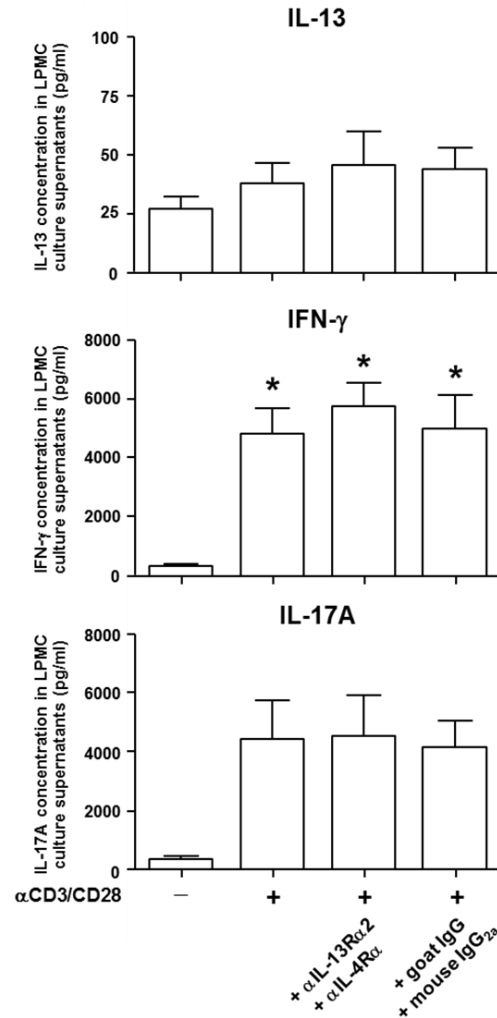


Figure 4.22

Figure 4.22. Effect of IL-13R and IL-4R blockade on cytokine production by UC LPMCs. Levels of IL-13, IFN- γ and IL-17A, expressed in pg/ml, in the supernatants of LPMCs isolated from the inflamed colon of three UC patients and cultured for 48 hours with medium alone (-) or with anti-(α)CD3/CD28 antibodies (+), in the absence or presence of a polyclonal goat α IL-13R α 2 neutralising antibody and a monoclonal mouse α IL-4R α neutralising antibody or their control IgG (goat IgG and mouse IgG_{2a}). Bars represent mean \pm SEM. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test. * $p < 0.05$ versus unstimulated LPMCs.

4.3.14 Bioassay for the functional evaluation of rhIL-13, anti-IL-13R α 2 and anti-IL-4R α antibodies

IL-13 increases claudin 2 expression in T84 epithelial cells (Prasad *et al.* 2005). On this basis, in order to assess whether rhIL-13 and the IL-13R α 2 and IL-4R α neutralising antibodies used in our *in vitro* assays were functionally active, T84 cells were cultured with rhIL-13 in the presence of a polyclonal goat anti-IL-13R α 2 neutralising antibody and a monoclonal mouse anti-IL-4R α neutralising antibody or their respective control IgG, and we evaluated the expression of claudin 2 in the T84 cell lysates by immunoblotting (Fig. 4.23). Culture with rhIL-13 induced a significant ($p<0.05$) increase in claudin 2 expression compared to culture with medium alone. The addition of anti-IL-13R α 2 and anti-IL-4R α neutralising antibodies significantly ($p<0.01$) reduced the expression of claudin 2 in rhIL-13-stimulated T84 cells, whereas the addition of the control IgG did not affect the expression of claudin 2 in rhIL-13-stimulated T84 cells.

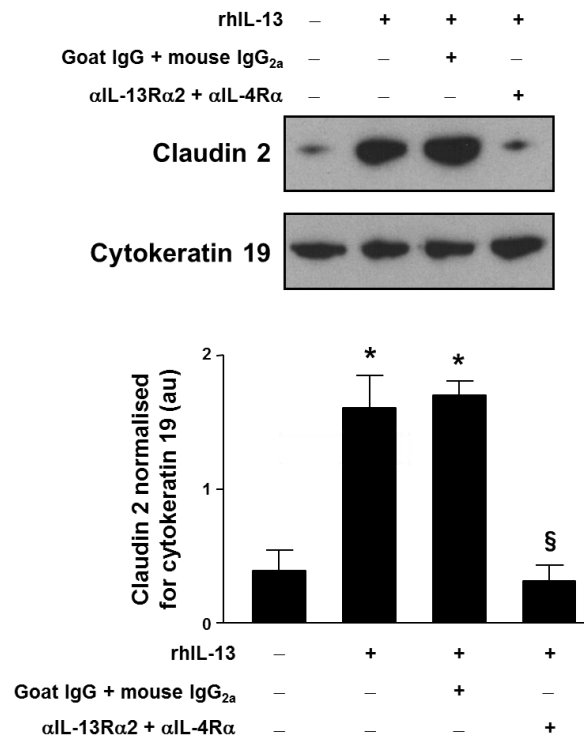


Figure 4.23 (see previous page). **Functional evaluation of rhIL-13, anti- (α)IL-13R α 2 and α IL-4R α antibodies.** Expression of claudin 2, detected by immunoblotting on the lysates of T84 cells cultured for 72 hours with medium alone or with rhIL-13, in the absence or presence of a polyclonal goat α IL-13R α 2 neutralising antibody and a monoclonal mouse α IL-4R α neutralising antibody or the respective control IgG (goat IgG and mouse IgG_{2a}). The example shown in the upper panel is representative of three separate experiments. Membranes were analyzed for cytokeratin 19 as a loading control for epithelial proteins. In the lower panel, densitometry of claudin 2 expression normalised for cytokeratin 19 is shown. Three independent repeats of the experiment were performed. Bars represent mean \pm SEM. au, arbitrary units. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test. * $p < 0.05$ versus T84 cells cultured with medium alone. $^{\S}p < 0.01$ versus T84 cells cultured with rhIL-13 and versus T84 cells cultured with rhIL-13 + control IgG.

4.3.15 Effect of IL-13 on pro-inflammatory cytokine production in UC

In order to assess the potential effects of IL-13 on the pro-inflammatory immune response observed in UC, unstimulated or anti-CD3/CD28-stimulated LPMCs from inflamed UC were cultured with rhIL-13, and the production of TNF- α , IFN- γ and IL-17A was measured by ELISA (Fig. 4.24). Stimulation with anti-CD3/CD28 antibodies significantly ($p < 0.05$) enhanced TNF- α , IFN- γ and IL-17A production by UC LPMCs (mean 4263 ± 705 pg/ml; mean 3977 ± 1030 pg/ml; mean 4983 ± 1236 pg/ml, respectively) compared to unstimulated conditions (mean 95 ± 14 pg/ml; mean 268 ± 92 pg/ml; mean 489 ± 129 pg/ml, respectively). The addition of rhIL-13 did not have any significant effect on TNF- α , IFN- γ and IL-17A production by both unstimulated (mean 97 ± 24 pg/ml; mean 254 ± 67 pg/ml; mean 413 ± 112 pg/ml, respectively) and anti-CD3/CD28-stimulated LPMCs (mean 4327 ± 292 pg/ml; mean 4537 ± 778 pg/ml; mean 5243 ± 1539 pg/ml, respectively).

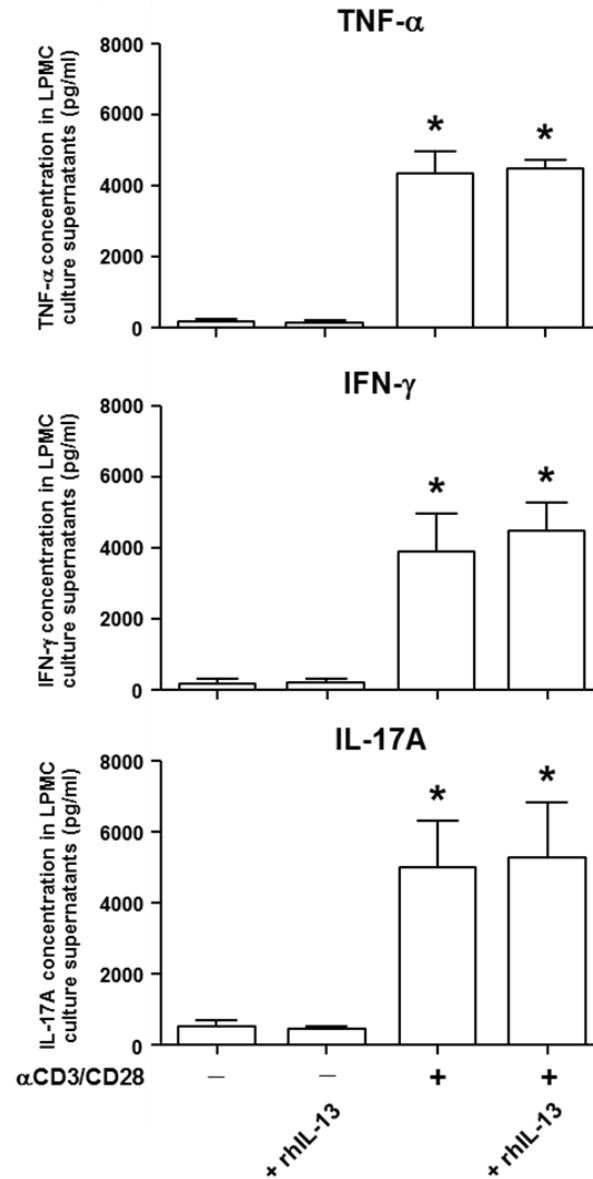


Figure 4.24

Figure 4.24. Effect of rhIL-13 on cytokine production by UC LPMCs. Levels of TNF- α , IFN- γ and IL-17A, expressed in pg/ml, in the supernatants of LPMCs isolated from the inflamed colon of three UC patients and cultured for 48 hours with medium only (-) or with anti- (α)CD3/CD28 antibodies (+), in the absence or presence of 20 ng/ml rhIL-13. Bars represent mean \pm SEM. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test. * $p < 0.05$ versus LPMCs cultured with medium only or with rhIL-13.

4.3.16 Effect of IL-13 on collagen production in CD

Finally, submucosal myofibroblasts isolated from uninfamed areas of strictured and non-strictured ileum of 5 patients with fibrostenosing CD, and from normal ileum of 5 control subjects, were cultured with rhIL-13, rhTNF- α or rhIL-13 + rhTNF- α , and collagen was measured in the culture supernatants (Fig. 4.25). Myofibroblast stimulation with rhIL-13 did not induce any significant change in mean collagen production compared to culture with medium alone (control myofibroblasts: 153 ± 38 μ g/ml *versus* 144 ± 26 μ g/ml, respectively; non-strictured CD myofibroblasts: 200 ± 55 μ g/ml *versus* 175 ± 47 μ g/ml, respectively; strictured CD myofibroblasts: 374 ± 115 μ g/ml *versus* 336 ± 124 μ g/ml, respectively). Compared to culture with medium alone, stimulation of strictured CD myofibroblasts with both rhTNF- α or rhIL-13 + rhTNF- α induced a significant increase in mean collagen production (804 ± 154 μ g/ml and 814 ± 153 μ g/ml, respectively). Compared to culture with medium alone, stimulation of control and non-strictured CD myofibroblast with both rhTNF- α or rhIL-13 + rhTNF- α induced an increase in mean collagen production (control myofibroblasts: 310 ± 63 μ g/ml and 335 ± 70 μ g/ml, respectively; non-strictured CD myofibroblasts: 390 ± 70 μ g/ml and 389 ± 74 μ g/ml, respectively), which however did not reach statistical significance. No significant difference in collagen production was found between myofibroblasts cultured with rhTNF- α and rhIL-13 + rhTNF- α . Strictured CD myofibroblasts cultured with rhTNF- α or rhIL-13 + rhTNF- α produced significantly ($p < 0.05$) more collagen than control myofibroblasts cultured in the same conditions.

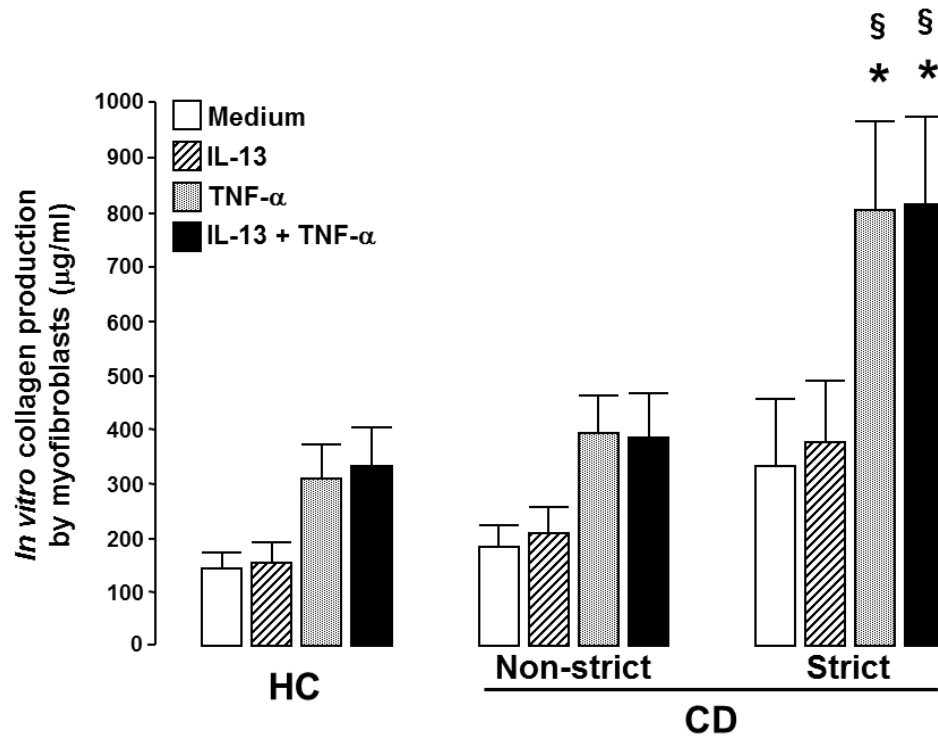


Figure 4.25

Figure 4.25. Effect of rhIL-13 on collagen production by intestinal myofibroblasts. Levels of collagen, expressed as $\mu\text{g/ml}$, in the supernatants of submucosal myofibroblasts isolated from uninflamed areas of strictured (Strict) and non-strictured (Non-strict) ileum of 5 patients with fibrostenosing CD, and from normal ileum of 5 control subjects (HC), cultured for 24 hours with medium alone or 20 ng/ml rhIL-13 or 20 ng/ml rhTNF- α , or 20 ng/ml rhIL-13 + 20 ng/ml rhTNF- α . Bars represent mean \pm SEM. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test. * $p < 0.05$ versus Strict myofibroblasts cultured with medium alone; § $p < 0.05$ versus HC myofibroblasts cultured in the same condition.

4.4 Results summary

In the current study, we found that:

1. IL-13 is not produced in excess by both mucosal explants and LPMCs from inflamed colon of UC patients in comparison to inflamed colon of CD patients and normal colon of control subjects.
2. NKT cells are reduced in the inflamed colonic mucosa of clinically active UC patients compared with NKT cells found in CD and control subjects, and their production of IL-13 is not up-regulated in UC inflamed colon.
3. Explants from strictured CD ileum produce small amounts of IL-13, not different from non-strictured CD or normal ileum.
4. Surface expression of IL-4R α , IL-13R α 1 and IL-13R α 2 on IBD and control CD3⁺ and CD68⁺ LPMCs is very low or not detectable. CD3⁺ and CD68⁺ LPMCs from inflamed CD mucosa contain high levels of IL-13R α 1.
5. Surface expression of IL-4R α , IL-13R α 1 and IL-13R α 2 on CD and control submucosal ileal myofibroblasts is very low or not detectable. Myofibroblasts from strictured CD ileum express significantly higher intracellular levels of IL-4R α , IL-13R α 1 and IL-13R α 2 than myofibroblasts isolated from control ileum.
6. IL-13 has no significant effects on the production of pro-inflammatory cytokines by colonic UC LPMCs.
7. IL-13 has no significant effects on the production of collagen by ileal CD and control myofibroblasts.

4.5 Discussion

Our findings indicate that IL-13 is not up-regulated in UC intestinal mucosa, and it is unlikely to play a functional role in the mucosal pro-inflammatory response in UC. Moreover, our results indicate that IL-13 is not up-regulated in CD intestinal strictures, and it is unlikely to contribute to intestinal fibrosis in this condition. This Section focuses on the interpretation of our findings in the context of the existing literature, on the limitations of our study, and on possible future experiments aimed at clarifying the involvement of IL-13 in disease processes in IBD patients.

IL-13 expression and production in intestinal inflammation

IL-13, which has 25% homology with IL-4, is released by Th2 and NKT cells and can also be expressed by innate cells including ILCs, eosinophils, basophils, and mast cells (van Dieren *et al.* 2007; Mannon *et al.* 2012). IL-13 expression is regulated at the transcriptional level by the classical Th2 transcription factor GATA3, and its production can be stimulated by epithelial-derived molecules including IL-33, IL-17E and TSLP (Brandt *et al.* 2011). IL-13 signals upon binding two transmembrane receptors, namely IL-13R α 2 or the heterodimer formed by IL-13R α 1 and IL-4R α (Rahaman *et al.* 2002; LaPorte *et al.* 2008). Upon binding the IL-13R α 1/IL-4R α heterodimer, IL-13 signals mostly through STAT6 activation, although other pathways may be activated, such as MAPK and phosphatidylinositol 3-kinase (PI3K). Following IL-13R α 2 binding, it has been considered that no intracellular signalling occurs, however it has been shown that STAT3 and MAPK activation can take place (Mannon *et al.* 2012). Interestingly, IL-13 binds IL-13R α 2 with higher affinity compared to the IL-13R α 1/IL-4R α heterodimer, and it is then rapidly internalised, therefore IL-13R α 2 has been traditionally considered as a scavenger or a decoy receptor for IL-13 (Kasaian *et al.* 2011). Moreover, IL-13R α 2 is mostly expressed as an intracellular protein and can be rapidly mobilised on the cell surface by IFN- γ , suggesting that Th1 immune response can negatively regulate IL-13 signalling (Konstantinidis *et al.*

2008). It has been shown that a soluble form of IL-13R α 2 exists, which can be generated by alternative splicing in mice, and by MMP-8 cleavage of the extracellular domain in humans (Chen *et al.* 2009).

IL-13 is part of type 2 immunity and plays an important role in counteracting helminth infections, in suppressing Th1-mediated autoimmunity, and in regulating wound repair mechanisms (Wynn 2015). Dysregulated IL-13 expression has been shown to play an important role in the pathogenesis of airway and skin disease, especially in asthma and atopic dermatitis (Ong 2014; Gour *et al.* 2015). The most important effects of IL-13 in asthma include goblet cell hyperplasia and hypersecretion, fibroblast activation, bronchial hyperresponsiveness and IgE class switch (Corren 2013). It has been shown that both recruitment of eosinophils and, more importantly, aeroallergen-induced airway resistance depend on IL-13R α 1 signalling in mice exposed to *Aspergillus* (Rothenberg *et al.* 2011). Excessive serum IL-13 has been reported in a subgroup of patients with atopic dermatitis associated with bronchial hyperresponsiveness (Lee *et al.* 2016).

In the gastrointestinal tract, IL-13 plays an important role in mediating the immune response against helminth infections (Grencis *et al.* 2004). In particular, clearance of nematodes from the intestine is dependent on IL-13R α 1 signalling (Sun *et al.* 2016). Excessive oesophageal expression of IL-5, IL-13 and IL-13-induced chemokines such as eotaxin-3 has been reported in patients with eosinophilic oesophagitis (Blanchard *et al.* 2011). Moreover, IL-5 and IL-13 are up-regulated in plasma of children with eosinophilic oesophagitis and food allergy (Frischmeyer-Guerrerio *et al.* 2011). Based mainly on results obtained on oxazolone-induced colitis, on the observation that IL-13-producing NKT cells are markedly increased in UC but not in CD inflamed mucosa, and on the existence of biologically plausible effects exerted *in vitro* by IL-13, such as the pro-apoptotic effect on IECs and the enhancement of epithelial permeability, IL-13 has been implicated in the pathogenesis of mucosal inflammation in UC (Heller *et al.* 2002;

Fuss *et al.* 2004; Heller *et al.* 2005). In particular, UC mucosal inflammation has been considered to be mediated by an atypical Th2 response, characterised by selective up-regulation of IL-13 and IL-5 in the absence of IL-4 up-regulation. This is consistent with an innate cell source of IL-13 and IL-5, as opposed to a classical Th2 response with overexpression of IL-4 (Mannon *et al.* 2012). There are, however, contrasting evidences on the role of IL-13 in intestinal immunity and inflammation. It has been shown in mice that both IL-4 and IL-13 play an important role in inducing intestinal goblet cell hyperplasia and mucin secretion, thereby promoting protective immunity against gastrointestinal helminths (Oeser *et al.* 2015), and goblet cell depletion is a classical histologic feature in inflamed UC mucosa. Furthermore, IL-13 plays a protective role in the *IL10*^{-/-} model of experimental colitis by suppressing Th1 and Th17 immune response via IL-13R α 1 signalling (Wilson *et al.* 2011). Finally, IL-13 stimulates epithelial cells to produce the anti-microbial RELM β , thereby protecting from nematode infections (Herbert *et al.* 2009).

In the present study, we found that the spontaneous production of IL-13 both by inflamed UC colonic biopsies cultured *ex vivo* for 24 hours and by unstimulated UC LPMCs cultured for 48 hours is low and not significantly different compared to that of CD patients and control subjects. Moreover, both IL-4 and IL-5 concentrations in our experiments were uniformly undetectable. Our findings are in keeping with those of Bernardo *et al.* (Bernardo *et al.* 2012), who observed nearly no production of IL-13 by both inflamed and uninflamed UC mucosa. In particular, it has been reported that organ culture supernatants from UC patients contain concentrations of IL-13 below the limit of detection in the majority of cases (10 out of 11 from uninflamed mucosa and 6 out of 11 from inflamed biopsies), and these did not correlate with clinical severity. IL-13 concentrations in inflamed UC organ culture supernatants were similar in our study (range 0-96 pg/ml) and in the study by Bernardo *et al.* (Bernardo *et al.* 2012) (range 0-approximately 85 pg/ml). Previous studies have also found significantly lower concentrations of IL-13 in intestinal organ culture supernatants and biopsy

homogenates of UC patients compared to CD patients (Vainer *et al.* 2000; Kadivar *et al.* 2004). Finally, it has been shown by immunohistochemistry that IL-13 expression is low or undetectable in active UC and control subjects, and it is low and limited to IECs in luminal CD (Scharl *et al.* 2013).

We observed that IL-13 transcripts in inflamed UC and CD mucosa were not significantly different from control mucosa. Polymorphisms in *IL-13* gene which alter the affinity for IL-13Rs have been reported in other diseases. In particular, the *IL-13* variant Arg130Gln, which produces an IL-13 peptide with lower affinity for the IL-13R α 2, has been found to be associated with asthma, atopy and systemic sclerosis (Vladich *et al.* 2005; Granel *et al.* 2006). However, the presence of this polymorphism has not been reported in UC (Waterman *et al.* 2006). It has been shown that intestinal IL-13 transcript levels are significantly lower in UC patients compared to control subjects (Verma *et al.* 2013). On the other hand, another study has reported that IL-13 mucosal transcripts are undetectable in control subjects, whereas 45% UC colonic samples tested are positive for IL-13 transcripts (Kawashima *et al.* 2011). Finally, mucosal transcripts for Th1, Th2 and Th17 cytokines have been investigated in inflamed ileal or colonic biopsies from UC and CD patients, and it has been shown that IL-21 and IL-13 display the largest difference in expression levels between the two diseases. However, the 5-marker set which has the highest discriminative power includes IL-21, IFN- γ , IL-12 p35, T-bet and GATA3, but does not include IL-13 (Iboshi *et al.* 2014).

Up-regulated expression of IL-4, IL-13 and IL-13R α 2 in CRC-derived cells is associated with liver invasion, metastasis and poor prognosis (Barderas *et al.* 2012). Moreover, tumour-infiltrating lymphocytes isolated from patients with colon cancer express both IL-4 and IL-13, which may play a role in invasive properties of cancer cells by down-regulating the expression of adhesion molecules (Kanai *et al.* 2000). It may be hypothesised that the overall lack of difference in IL-13 production between IBD patients and control subjects observed in our study may

derive from IL-13 up-regulation in samples from patients who underwent surgery for colon cancer. However, only part of the mucosal explants in our control group were collected from patients who underwent an intestinal resection for colon cancer, and samples were taken at least 50 cm far from the tumoral mass, which makes the possibility of collecting tumour-infiltrating lymphocytes unlikely. Moreover, samples used for the analysis of IL-13 transcript levels did not include patients undergoing surgery for colon cancer, and the results confirmed the absence of significant differences in IL-13 expression between control subjects and IBD patients.

Internalisation upon binding to IL-13R α 2 is an established mechanism responsible for the rapid clearance of IL-13, and exposure to IL-13 induces the up-regulation of IL-13R α 2, thereby amplifying its effects (Kawakami *et al.* 2001; Kasaian *et al.* 2011). We hypothesised that the low levels of IL-13 observed in our culture supernatants might depend on its binding to IL-13Rs and subsequent internalisation by LPMCs. We therefore performed anti-CD2/CD28 and anti-CD3/CD28-stimulations of UC LPMCs in the presence of blocking antibodies to IL-13R α 2 and IL-4R α , however this did not induce any significant change in IL-13 concentration in culture supernatants.

Both anti-CD2/CD28 and anti-CD3/CD28 stimulations of LPMCs from inflamed UC and CD or normal colon, despite inducing a marked increase in both IFN- γ and IL-17A, overall had no significant effect on IL-13 release, without significant differences in the levels of this cytokine between the three groups studied. Despite the conditions of our experiments being similar, particularly regarding anti-CD2/CD28 stimulation, in the present study we could not replicate the findings of Fuss *et al.* (Fuss *et al.* 2004) and Heller *et al.* (Heller *et al.* 2005). In particular, compared to the previous studies, while anti-CD2/CD28 stimulation resulted in a significant increase in IFN- γ production, without significant difference between CD and UC LPMCs, we did not observe an increase in IL-13 concentration in the supernatants of UC LPMCs cultured in the same conditions.

It is important to underline that, while anti-CD3/CD28 stimulation induced a clear up-regulation of IFN- γ and IL-17A production by LPMCs in all CD and UC patients and control subjects, the same stimulation largely failed to induce IL-13 release. However, a small, but clear increase in IL-13 production after anti-CD3/CD28 stimulation was present in LPMC supernatants from five out of 11 CD patients, five out of 11 UC patients and three out of 11 control subjects, suggesting that, upon T cell activation, IL-13 production may be up-regulated in a subset of IBD patients and control subjects. Nevertheless, when considering LPMCs which produced higher concentrations of IL-13 following anti-CD3/CD28 stimulation, the up-regulation reached a maximum concentration of <200 pg/ml, as opposed to a mean concentration around 1000 pg/ml measured in supernatants of LPMCs cultured with anti-CD2/CD28 antibodies in the previous studies (Fuss *et al.* 2004; Heller *et al.* 2005). Unfortunately, in the previous studies, cytokine concentrations in the supernatants of stimulated LPMCs were presented as means \pm SEM, therefore it is not possible to determine the relative contribution of each sample to the overall results, and to evaluate the heterogeneity between different patients (Fuss *et al.* 2004; Heller *et al.* 2005).

We can only speculate why we were not able to show enhanced IL-13 and IL-5 expression and production in UC compared to other studies (Del Zotto *et al.* 2003; Fuss *et al.* 2004; Heller *et al.* 2005; Kawashima *et al.* 2011). In particular, we believe that our experimental settings were adequate and appropriately sensitive in order to detect differential production of the cytokines examined in the different groups of patients studied. We were able to detect high production of IL-17A (>400 pg/ml in some IBD patients) and IFN- γ (>300 pg/ml in some IBD patients) in our 24 hour *ex vivo* organ cultures, and we observed high levels of IFN- γ (never <2000 pg/ml in IBD patients) and IL-17A (never <2000 pg/ml in IBD patients) in our 48 hour *in vitro* LPMC cultures. It should be noted that, when intestinal samples were collected, the large majority of CD and UC patients in our study were not on treatment with immunosuppressants, which profoundly

dampen cytokine secretion and may deplete T cell subsets by the induction of apoptosis. Conversely, immunosuppressants may have influenced the findings reported in other investigations. It has been shown that cyclosporine A, which is used intravenously as a rescue therapy in order to avoid colectomy in ASUC, induces T cell apoptosis and down-regulates IL-13 production by PBMCs isolated from UC, but not CD, patients (Steiner *et al.* 2015). In our study, however, none of the patients were on treatment or had been previously treated with cyclosporine A. Mucosal transcripts for IL-4, IL-5, and IL-13, have been reported to be increased compared to control subjects during all stages of CD (Zorzi *et al.* 2013). We included patients with both early- and late-stage disease, and, while IFN- γ production by LPMCs seemed more prominent in early CD, we did not find any correlation or trend between disease duration and IL-13 production.

Biopsies, unstimulated LPMCs and stimulated LPMCs from inflamed colon of both CD and UC patients produced significantly higher amounts of IFN- γ and IL-17A than those from uninfamed UC, CD and control subjects, with no significant differences between CD and UC. This is a major discrepancy between our study and previous ones, which showed high IFN- γ expression and production by activated CD LPMCs, as opposed to significantly lower IFN- γ expression and release by activated UC and control LPMCs (Fuss *et al.* 2004; Heller *et al.* 2005; Sarra *et al.* 2010). Nonetheless, also in previous studies IFN- γ concentration in supernatants of anti-CD2/CD28-stimulated UC LPMCs was around 2000 pg/ml, whereas that of IL-5 was 150-200 pg/ml and that of IL-13 around 1000 pg/ml, highlighting the relative importance of IFN- γ compared to Th2 cytokines (Fuss *et al.* 2004; Heller *et al.* 2005). While differences between ours and the findings by Fuss *et al.* (Fuss *et al.* 2004) and Heller *et al.* (Heller *et al.* 2005) are difficult to explain since the experimental conditions are very similar, PMA plus ionomycin stimulation used in the study by Sarra *et al.* (Sarra *et al.* 2010) may induce different T cell responses. Moreover, we previously reported no difference between CD and UC in terms of IFN- γ -producing T-LPLs after stimulation with PMA plus ionomycin (Rovedatti *et al.* 2009). Determinations performed using

different methods may lead to different results, and indeed in our qRT-PCR determination we could observe that IFN- γ expression is higher in CD than in UC and control subjects, however post-transcriptional modifications exist and may lead to discrepancy between mRNA and protein levels (Washburn et al. 2003; Laloo et al. 2009), which in turn may be responsible for the lack of difference in IFN- γ protein levels between CD and UC patients.

Unlike IFN- γ , we found IL-17A to be similarly up-regulated in CD and UC as in the previous studies (Rovedatti *et al.* 2009; Sarra *et al.* 2010). Nevertheless, the magnitude of IL-17A up-regulation which we observed in supernatants from CD and UC inflamed colonic biopsies compared to those from uninfamed IBD biopsies was less than 2 folds, which raises the question whether this statistically significant difference has a biological relevance in IBD. In the study by Bernardo *et al.* (Bernardo *et al.* 2012), IFN- γ , IL-4, IL-6, IL-7 and TNF- α , but not IL-17A, concentrations were all significantly higher in organ culture supernatants from inflamed UC mucosa compared to uninfamed UC mucosa, however IL-4 concentrations were below 7.5 pg/ml in all cases tested, and the only cytokine which correlated with the endoscopic Mayo score was IL-6. Interestingly, it has been shown that serum IL-17A, TNF- α and IL-6, but not IFN- γ , correlate both with CAI and with the endoscopic Mayo score in UC (Mańkowska-Wierzbicka *et al.* 2015).

IL-13-producing cells in intestinal inflammation

We observed a significantly reduced percentage of NKT cells, defined as CD3⁺CD161⁺ cells, among T-LPLs from the inflamed colon of UC patients compared to the inflamed colon of CD patients and to the normal colon of control subjects. Our observation confirms the findings by Shimamoto *et al.* (Shimamoto *et al.* 2007), who also observed that CD161⁺ T cells are significantly decreased in the inflamed mucosa of UC patients compared to control subjects. Even upon stimulation with anti-CD3/CD28, the intracellular production of IL-13 by mucosal

NKT cells was low in all the groups studied. This is different from the studies by Fuss *et al.* (Fuss *et al.* 2004; Fuss *et al.* 2014), who reported that up to 24% lamina propria NKT cells in UC release IL-13, and that more than 30% LPMCs in UC is constituted by non-invariant NKT cells which produce IL-13 upon stimulation with lyso-sulfatide-loaded CD1d-tetramer.

In keeping with Fuss *et al.* (Fuss *et al.* 2004), we observed that there are no mucosal iNKT ($CD3^+V\alpha24^+V\beta11^+$) cells in UC. Conversely, in contrast to Fuss *et al.* (Fuss *et al.* 2004), who could not detect any lamina propria $CD3^+V\alpha24^+V\beta11^-$ cells, we report the presence of a small but definite population of $CD3^+$ LPMCs expressing $V\alpha24$, as would be expected for a commonly used α chain, which was not significantly increased in UC patients compared to CD patients and control subjects. Since we were mostly interested in iNKT cells, we chose the plots with the most representative iNKT cell populations, and since iNKT in UC were very low ($<0.1\%$ $CD3^+$ LPMCs) we acquired more events ($>50,000$), hence $CD3^+V\alpha24^+V\beta11^-$ LPMCs seem more frequent in UC, but percentage-wise this is not the case. The mean percentage of $CD3^+V\alpha24^+V\beta11^-$ LPMCs in CD was higher, although not significantly, compared to UC and control LPMCs, however we only evaluated this on three patients per group, which limits the possibility to draw any conclusion, particularly with regards to positive observations. Moreover, the high mean percentage of $CD3^+V\alpha24^+V\beta11^-$ LPMCs observed in CD was due to a single patient with 47% $CD3^+V\alpha24^+V\beta11^-$ LPMCs, probably because of the random oligoclonality seen in intestinal T cells, where only a few clones make up the repertoire, in whom a dominant clone expressed $V\alpha24$.

In vitro effects of IL-13 in intestinal inflammation

The previous experiments are descriptive and suggest that IL-13 is not up-regulated in the inflamed mucosa of the majority of UC patients, and that IL-13-producing cells are a small proportion of mucosal immune cells in UC, at least in the patients examined. It is nevertheless important to remember that IL-13 has

important *in vitro* effects in the intestine. It has been shown *in vitro* using HT-29 cells that IL-13 induces epithelial cell apoptosis, inhibits restitution of epithelial cell monolayers, and disrupts epithelial permeability (Heller *et al.* 2005). Furthermore, it has been observed that IL-13 activates the pro-apoptotic protease caspase-3 in mouse colonic IECs (Kawashima *et al.* 2011), and that IL-13 enhances epithelial permeability by up-regulating claudin 2 expression within the tight junctions (Weber *et al.* 2010). We performed functional experiments on LPMCs from inflamed UC mucosa. In particular, the addition of rhIL-13 to unstimulated or anti-CD3/CD28-stimulated LPMCs from inflamed colonic UC mucosa did not have any influence on their production of classical pro-inflammatory cytokines such as TNF- α , IFN- γ and IL-17A. In accordance with our results, it has been shown that culturing for 24 hours *ex vivo* UC biopsies from inflamed colon with anti-IL-13 antibody, or UC biopsies from uninfamed colon with rhIL-13 does not induce any significant difference on pro-inflammatory and regulatory cytokine concentration in culture supernatants (Bernardo *et al.* 2012). This observation is particularly interesting, since in the same study it has been reported that organ culture of uninfamed or inflamed UC intestinal biopsies with rhIL-6 or anti-IL-6 antibody, respectively, does influence the cytokines released in the supernatants, promoting a pro-inflammatory or an anti-inflammatory microenvironment, respectively. This suggests that the organ culture model used is responsive to exogenous stimuli and provides the opportunity to detect the effects of one specific cytokine or its neutralisation. One possible reason to explain our results is that rhIL-13, IL-13R α 2 and IL-4R α neutralising antibodies used in our *in vitro* experiments were not biologically active, however this is not the case, as shown by the ability of anti-IL-13R α 2 and anti-IL-4R α antibodies to inhibit rhIL-13-induced claudin 2 up-regulation in T84 cells.

IL-13R expression in intestinal inflammation

It has been shown that both IL-13R α 1 and IL-4R α are expressed on the surface of HT-29 cells and IECs of UC patients and control subjects, and are responsible

for the effects of IL-13 on epithelial apoptosis and permeability (Heller *et al.* 2005). It has subsequently been observed that lamina propria NKT cells in UC express high levels of IL-13R α 2 (Fuss *et al.* 2014). We therefore studied IL-13R expression by different LPMC subtypes in inflamed IBD and control mucosa. In particular, we assessed the expression of IL-4R α , IL-13R α 1 and IL-13R α 2 by T cells (CD3⁺ LPMCs) and macrophages (CD68⁺ LPMCs) from normal mucosa of control subjects and from inflamed UC and CD mucosa. We observed that IL-4R α , IL-13R α 1 and IL-13R α 2 expression profile is similar between intestinal mucosal T cells and macrophages. Surface expression of IL-4R α , IL-13R α 1 and IL-13R α 2 by both T cells and macrophages from normal mucosa of control subjects and from inflamed UC and CD mucosa was low or not detectable. We hypothesised that the low surface expression of IL-13Rs by LPMCs could derive from the removal of receptors by collagenase digestion during LPMC isolation. In order to investigate this, we repeated the same staining after overnight culture of freshly isolated LPMCs within low-adherence tissue culture plates, however the expression of IL-13Rs was not modified.

Since IL-13R α 2 is present mostly within the cytoplasm (Konstantinidis *et al.* 2008), we evaluated the levels of IL-13Rs in mucosal immune cells in IBD and control subjects by flow cytometry upon intracellular staining. We added the anti-IL-4R α , anti-IL-13R α 1 and anti-IL-13R α 2 antibodies after LPMC permeabilisation, and, since the surface expression of the receptors on the same cells was low or not detectable, we considered any positive signal as being the result of intracellular expression. The intracellular expression of IL-4R α , IL-13R α 1 and IL-13R α 2 by intestinal T cells and macrophages from normal mucosa of control subjects and from inflamed UC mucosa was low or not detectable. Intestinal T cells and macrophages from inflamed CD mucosa, which expressed low levels of IL-4R α and IL-13R α 2, contained high levels of IL-13R α 1. It has been shown that non-responsiveness to infliximab treatment in UC, defined as lack of endoscopic and histologic healing, is associated to significantly higher

mucosal expression of IL-13R α 2 (Arijs *et al.* 2009), which is difficult to interpret, since TNF- α is an inducer of IL-13R α 2, and higher IL-13R α 2 expression should dampen the effects of IL-13. Collectively, our data on IL-13R expression in UC are in keeping with our results showing the lack of pro-inflammatory effects of rhIL-13 on UC LPMCs.

IL-13 and IL-13Rs in intestinal fibrosis

IL-13 has an important role in experimental fibrosis and in diseases characterised by tissue fibrosis (Lee *et al.* 2001; Fichtner-Feigl *et al.* 2008a; Fichtner-Feigl *et al.* 2008b). In particular, IL-13 has been shown to play an important pro-fibrogenic role in systemic sclerosis, asthma, and idiopathic pulmonary fibrosis (Wilson *et al.* 2009; Fuschiotti 2011; Agrawal *et al.* 2014). IL-13 is thought to exert its pro-fibrogenic effects by stimulating the production of TGF- β by macrophages via IL-13R α 2 activation, and this mechanism has been shown to be responsible for intestinal fibrosis in oxazolone- and in chronic TNBS-induced colitis (Fichtner-Feigl *et al.* 2006; Fichtner-Feigl *et al.* 2008b). However, intestinal stricture formation is rare in UC, and the involvement of IL-13 in CD intestinal fibrosis is unclear.

We observed that muscle layer explants from strictured CD ileum release small amounts of IL-13, not significantly different from non-strictured CD or normal ileum. Conversely, IL-1 β and collagen levels in the supernatants and TGF- β 1 transcripts in the homogenates of cultured explants were significantly higher in strictured CD compared to non-strictured CD or normal ileum. Our data are in contrast with Bailey *et al.* (Bailey *et al.* 2012), who observed that IL-13 mRNA is overexpressed in the lysates of fibrotic intestinal muscle of CD patients compared to control subjects. Post-transcriptional modifications may explain, at least in part, this discrepancy (Washburn *et al.* 2003; Laloo *et al.* 2009). Interestingly, it has been shown that IL-13 is highly expressed by transitional

cells lining fistulae in CD, and that IL-13 induces genes involved in cell invasion, which are likely to contribute to the formation of fistulae (Scharl *et al.* 2013).

We observed that the surface and intracellular expression of IL-4R α , IL-13R α 1 and IL-13R α 2 by both T cells and macrophages from the mucosa overlying CD strictures and CD mucosa above non-strictured areas is low or not detectable. Furthermore, surface expression of IL-4R α , IL-13R α 1 and IL-13R α 2 on submucosal myofibroblasts isolated from uninflamed areas of strictured and non-strictured ileum of patients with fibrostenosing CD, and from normal ileum of control subjects was low or not detectable. In order to exclude that the low surface expression of IL-13Rs by submucosal myofibroblasts could derive from the removal of receptors by trypsin during the passage of cells, we repeated the same staining after overnight culture of freshly passaged myofibroblasts within low-adherence tissue culture plates, and we observed that the expression of IL-13Rs was not modified. Conversely, compared to myofibroblasts isolated from control ileum, myofibroblasts isolated from uninflamed areas of strictured CD ileum expressed detectable and significantly higher intracellular levels of IL-4R α , IL-13R α 1 and IL-13R α 2, and myofibroblasts isolated from uninflamed areas of non-strictured CD ileum expressed detectable and significantly higher intracellular levels of IL-13R α 1 and IL-13R α 2. It has been shown that myofibroblast-like transitional cells lining CD fistulae and epithelial-like cells close to the fistulae express high levels of IL-13R α 1 (Scharl *et al.* 2013). Another study has reported that IL-13R α 1, but not IL-13R α 2, is highly expressed by smooth muscle cells and ILCs infiltrating CD intestinal strictures (Bailey *et al.* 2012).

Functional data on experimental intestinal fibrosis support an important role for IL-13, and there are several evidences that blocking IL-13 signalling at different levels improves intestinal fibrosis in mice. It has been shown that the PPAR γ activator GED-0507-34 Levo ameliorates chronic DSS-induced intestinal fibrosis, and this is associated with a significant down-regulation of mucosal IL-13 (Specia *et al.* 2016). However, it is unclear whether IL-13 down-regulation plays an active

role in the improvement of fibrosis. Indeed, it has been shown that the small molecule HSc025, which antagonises TGF- β signalling, improves TNBS-induced intestinal fibrosis despite not having any effects on the mucosal levels of IL-13 and TNF- α (Imai *et al.* 2015).

Since IL-13 and TNF- α can exert a synergistic effect on the expression of IL-13R α 2, and TNF- α has a known pro-fibrogenic action (Fichtner-Feigl *et al.* 2006), we finally performed a functional experiment *in vitro* by testing the effect of exogenous rhIL-13 and/or rhTNF- α on submucosal myofibroblasts isolated from strictured CD ileum and control ileum. As opposed to TNF- α , which indeed increased the production of collagen, no direct effect was exerted by IL-13 on collagen release by CD and control myofibroblasts, in keeping with existing data (Bailey *et al.* 2012). It has to be noted that Bailey *et al.* performed *in vitro* experiments on fibroblasts isolated from the muscle layer (Bailey *et al.* 2012), whereas we studied submucosal myofibroblasts. This may provide a possible explanation for the different effect of TNF- α on collagen production which we observed in our experiments.

Limitations of the study

Unfortunately, when working with human tissue, sample size can often be a relevant limitation. In our study, this affected mainly the experiments regarding IL-13R expression in intestinal cells from IBD patients and control subjects, and *in vitro* effects of IL-13 on UC LPMCs, for which only very few samples could be analysed (n=3). This needs to be taken into account in the interpretation of the results, particularly in the case of the up-regulated IL-13R α 1 expression in T cells and macrophages from inflamed CD mucosa, and in the case of the higher intracellular levels of IL-4R α , IL-13R α 1 and IL-13R α 2 in CD myofibroblasts compared to myofibroblasts isolated from control ileum. With a very small sample size, particularly the positive results need to be interpreted with caution, as both interindividual and intraindividual variability are more likely to have a relevant

impact on the data, and should be validated in a larger cohort of patients before any conclusion can be made.

Organ culture of intestinal biopsies has not been formally validated, however it has been recognised as a useful technique to investigate the abnormal immune response in IBD, as it recapitulates the architecture and organisation of the intestinal mucosa with the use of human tissue. Relevant shortcomings of the organ culture approach, particularly when compared to organoid generation and culture, are the limited time (maximum 36 hours) during which the tissue remains viable, and sample availability. One possible limitation of the organ culture system is that, in addition to the interindividual variability in the severity of mucosal inflammation and to the intraindividual variability due to heterogeneity in the severity of mucosal inflammation in different sections of the colon or the small intestine, there is additional variability due to the different size of biopsy samples. This, however, can be minimised by normalising the results by weight. As an example of the potential validity and predictive value of the organ culture model, the Smad7 antisense oligonucleotide inhibitor mongersen, which has been shown promising efficacy data in CD, was first tested on CD biopsies using the organ culture system (Monteleone *et al.* 2001; Monteleone *et al.* 2015). We are currently assessing the potential efficacy of novel molecules for the treatment of IBD using the organ culture system. In addition to organ culture, in our study we have assessed IL-13 expression and production in IBD inflamed mucosa using other methods, including qRT-PCR and LPMC isolation and culture, and the results were consistent with those obtained using organ culture.

For the *in vitro* experiments, when available, we used reagents at concentrations based upon previous data existing in the literature (e.g. we used rhIL-13 and rhTNF- α at 20 ng/ml, which is the same concentration used by Fichtner-Feigl *et al.* 2006). When no previous results were available, we decided the concentrations of the reagents based on the reagent data sheet, as in the case of anti-IL-4R α and anti-IL-13R α 2 antibodies. The concentration of rhIL-13 that we

used in our *in vitro* experiments (20 ng/ml) is ten-fold higher than that reported to induce changes in epithelial permeability, and the same described to exert pro-fibrogenic effects, so we have no doubt that we added active doses to the LPMCs (Kadivar *et al.* 2004; Prasad *et al.* 2005), and this is confirmed by the up-regulation of claudin 2 expression induced by rhIL-13 in T84 cells. However, we are unable to draw any conclusion on the physiological relevance of this concentration *in vivo*, and there is the possibility that this is excessive compared to the *in vivo* levels in the intestinal mucosa, especially as IL-13 concentrations in culture supernatants were never >200 pg/ml. However, it is important to note that cytokines act locally, therefore the physiologically relevant concentration is the one present in close proximity of the target cells, and our data are not informative regarding this.

Future experiments

There is evidence of the importance of IL-13 in experimental models of colitis and intestinal fibrosis, however there is poor correlation between murine and human genomic response to acute inflammatory stress (Seok *et al.* 2013). Indeed, our results suggest that it is hard to envisage a prominent role for this cytokine in the overall population of patients with UC and fibrostenosing CD. Several experiments are needed to address specific aspects of IL-13 involvement in the gastrointestinal mucosal immune response, and to investigate the possibility that IL-13 contributes to the disease process in a subset of IBD patients.

Our results on IL-13 expression and production in the intestinal mucosa were obtained using qRT-PCR and ELISA, however other methods such as immunohistochemical characterisation would be useful to corroborate the data, and should be used in a future validation study. Immunohistochemical characterisation of IL-13Rs would also be needed to draw more informative conclusions on the expression levels in the intestinal mucosa of the different groups studied, particularly considering the previous studies on this aspect

(Bailey *et al.* 2012; Scharl *et al.* 2013).

It has been shown that a specific subset of ILCs, namely type 2 ILCs (ILC2), depend on GATA3 for their development and display a cytokine production profile characterised by the release of high amounts of IL-5, IL-13, and relatively small amounts of IL-4 (Zhu 2015). ILC2 are involved in the early disease phase in allergic skin and lung inflammation (Monticelli *et al.* 2011; Roediger *et al.* 2013). The atypical Th2 cytokine profile expressed by ILC2 makes them a particularly interesting cell type to study in IBD, particularly in the early disease phase and in treatment-naïve patients.

Regarding the involvement of IL-13 in CD intestinal fibrosis, we did not study the effects of IL-13 on the production of tissue remodelling enzymes such as MMPs and TIMPs by intestinal cells contributing to the process of stricture formation. This would be particularly important, as it has been shown that *in vitro* culture with IL-13 down-regulates spontaneous MMP-2 expression and TNF- α -induced MMP-1 and MMP-9 synthesis by intestinal smooth muscle CD fibroblasts (Bailey *et al.* 2012). On the other hand, it would be particularly interesting to explore the effects of MMPs on IL-13 signalling in the intestinal mucosa, as MMP-8 cleavage of the extracellular domain is responsible for the generation of soluble IL-13R α 2 in humans (Chen *et al.* 2009).

Finally, using the IMPACT IL-13 assay, which has high sensitivity – in the range of fg/ml – and specificity, it has been reported that sera of patients with asthma, atopic dermatitis and idiopathic pulmonary fibrosis, have significantly higher concentrations of IL-13 compared to control sera (Cai *et al.* 2016). It would be interesting to study whether mucosal expression of IL-13 correlates with serum concentrations of IL-13 in UC, in order to identify by means of a simple biomarker test the subset of patients in whom this cytokine is more likely to play a pathogenic role.

Chapter 5: Effects of MMP-3, MMP-9 and MMP-12 on the integrity and function of anti-TNF- α agents in IBD

Information on the bioavailability of anti-TNF- α agents in inflamed tissues, where they are expected to exert their effect, is lacking. MMPs are up-regulated in IBD inflamed mucosa, and can cleave human IgG₁ near the hinge region. Anti-TNF- α agents in use in clinical practice are IgG₁ or have part of the amino acid sequence in common with Ig. Further information on the background to this Chapter is reported in Sections “1.4.5 TNF- α and TNF- α neutralisation in IBD” and “1.4.6 Proteases in IBD intestinal mucosa”.

We have studied MMP-3 and MMP-12 due to their up-regulation in intestinal inflammation and to the fact that they can specifically cleave IgG₁ at the same point in the lower hinge, and MMP-9 as it is one of the main proteolytic enzymes associated to intestinal inflammation.

5.1 Aims of the study

This study aims to explore the effects of MMPs on the integrity and function of anti-TNF- α agents in the context of IBD, and to evaluate whether MMP degradation may be relevant in primary non-responsiveness to biologic therapy in IBD. We have used the following approach:

1. Evaluation of the effects of MMP-3, MMP-9 and MMP-12 on the integrity and TNF- α neutralisation function of anti-TNF- α agents.
2. Assessment of the effects of protein extracts from inflamed IBD mucosa on the integrity and TNF- α neutralisation function of anti-TNF- α agents.
3. Study of serum levels of MMP-3-/MMP-12-cleaved anti-TNF- α agents in IBD patients.
4. Study of serum levels of MMP-3-/MMP-12-cleaved endogenous IgG and anti-hinge autoantibodies in IBD patients and control subjects.

5.2 Chapter-specific Materials and Methods

5.2.1 Patients and tissues

In order to assess the effect of proteins from human intestinal mucosa on the integrity and function of TNF- α neutralising agents, perendoscopic biopsies from the inflamed colon of eight CD and eight UC patients (Table 5.1) and from the normal colon of eight control subjects (Table 5.2) were collected and processed for protein extraction. Sera from 29 active CD and 33 active UC patients before and after infliximab treatment, and from 20 control subjects were collected. Responsiveness to infliximab treatment was defined as the concomitant presence of clinical remission and complete mucosal healing (Levesque *et al.* 2015) at the first endoscopic examination after the treatment start. This study was approved by the NRES Committee London - City & East (REC reference: 10/H0704/74), and informed consent was obtained from all IBD patients and control subjects from whom biopsies were collected. IBD sera were obtained from Professor Severine Vermeire's clinic (Department of Gastroenterology, University Hospital Gasthuisberg, Leuven, Belgium).

Table 5.1. Clinical features of IBD patients (n=16)

	CD (n=8)	UC (n=8)
Median age – years (range)	28 (20-55)	30.5 (22-63)
Male/Female (%)	5/3 (62.5/37.5)	4/4 (50/50)
Median duration of disease – years (range)	4 (0.5-11)	3 (0-15)
Extent of disease		
Ileocolon – CD (%)	4 (50)	NA
Colon – CD (%)	4 (50)	NA
Left-sided colitis – UC (%)	NA	5 (62.5)
Pancolitis – UC (%)	NA	3 (37.5)
Disease behaviour		
Non-stricturing non-penetrating – CD (%)	8 (100)	NA
Stricturing – CD (%)	0 (0)	NA
Penetrating – CD (%)	0 (0)	NA
Clinical disease activity		
Mild (%)	0 (0)	0 (0)
Moderate (%)	3 (37.5)	2 (25)
Severe (%)	5 (62.5)	6 (75)
Grading of histologic inflammation		
Mild (%)	0 (0)	0 (0)
Moderate (%)	0 (0)	1 (12.5)
Severe (%)	8 (100)	7 (87.5)
Treatment		
None	1 (12.5)	2 (25)
5-aminosalicylates (%)	2 (25)	4 (50)
Corticosteroids (%)	3 (37.5)	4 (50)
Immunosuppressants		
Azathioprine/6-mercaptopurine (%)	2 (25)	3 (37.5)
Methotrexate (%)	1 (12.5)	0 (0)
Corticosteroids + immunosuppressants (%)	3 (37.5)	3 (37.5)

CD, Crohn's disease; NA, not applicable; UC, ulcerative colitis.

Table 5.2. Clinical features of control subjects (n=8)

Characteristics and parameters	N.	Median (range)
Age (years)		34 (26-45)
Sex (M/F)	4/4	
Comorbidities		
<i>Hypertension</i>	2	
<i>None</i>	6	
Current medications		
<i>Anti-hypertensive</i>	2	
<i>Proton pump inhibitors</i>	2	
<i>None</i>	5	

F, female; M, male.

5.2.2 Study design

First, cleavage reactions with rhMMP-3, rhMMP-12, rhMMP-9 and infliximab, adalimumab, etanercept were performed in order to study the effect of MMPs on the integrity and soluble TNF- α neutralisation function of anti-TNF- α agents. Then, the possibility to prevent the effect of rhMMP-3 and rhMMP-12 on anti-TNF- α agents using MMP inhibitors was investigated. Protein extracts from normal colonic biopsies of eight control subjects and from inflamed colonic biopsies of 16 IBD patients were subsequently prepared and, upon measuring MMP-3/MMP-12 activity, cleavage reactions were performed in the absence or presence of MMP inhibitors in order to study the effect of protein extracts on the integrity and soluble TNF- α neutralisation function of anti-TNF- α agents. Then, experiments were performed in order to assess whether cleaved antibodies could be identified in the sera of IBD patients and control subjects. In particular, ELISA assays aimed at detecting MMP-3-/MMP-12-cleaved anti-TNF- α agents, MMP-3-/MMP-12-cleaved endogenous IgG and anti-hinge autoantibodies were developed by testing sera of 29 active CD and 33 active UC patients either before (MMP-3-/MMP-12-cleaved endogenous IgG and anti-hinge autoantibodies) or after (MMP-3-/MMP-12-cleaved anti-TNF- α agents) infliximab therapy, and sera of 20 control subjects.

5.2.3 Protein extraction

Biopsies were placed in PBS (Oxoid Ltd, Basingstoke, UK) and homogenised by sonication. Endogenous IgG were removed from the mucosal protein extracts by two cycles of Protein G SpinTrap (GE Healthcare, Little Chalfont, UK), and total protein concentration was normalised to 2 mg/ml by dilution in PBS.

5.2.4 MMP activity assay

MMP-3/MMP-12 activity in IBD and control mucosal protein extracts was measured using the fluorescence resonance energy transfer (FRET)-based Sensolyte[®] 520 MMP-12 assay kit from AnaSpec Inc. (Fremont, CA, USA). This kit uses a 5-FAM (fluorophore)- and QXL520[™] (quencher)-labeled FRET peptide. In an intact FRET peptide, the fluorescence of 5-FAM is quenched by QXL520[™]. Upon cleavage of the FRET peptide by MMP-3 or MMP-12 (the FRET peptide provided with this kit is cleaved by both MMP-3 and MMP-12), the fluorescence of 5-FAM is recovered. Protein extracts were incubated with the FRET peptide at 37°C for 1 hour and fluorescence was subsequently measured using (fluorescence detection mode, 490 nm/520 nm excitation/emission wavelengths) the Bio-tek Synergy[™] HT Multi-Detection Microplate Reader (BioTek). Active rhMMP-3 and rhMMP-12, each one at the concentration of 1 µg/ml, were used as positive controls.

5.2.5 Cleavage reactions

Upon activation, rhMMP-3 and rhMMP-12 were co-incubated with TNF- α neutralising agents in order to subsequently evaluate their effect on the drug integrity and function. In particular, rhpro-MMP-3 and rhpro-MMP-9 (both from R&D Systems), at a concentration of 50 µg/ml, were activated by 24 hour incubation at 37°C with p-aminophenylmercuric acetate (p-APMA; Sigma-Aldrich) 1 mM in 50 mM Tris, 10 mM CaCl₂, 150 mM NaCl, 0.05% Brij 35 (Sigma-Aldrich)

(TCNB) buffer, whereas rhpro-MMP-12 (R&D Systems) 50 µg/ml was auto-activated by 30 hour incubation at 37°C in TCNB buffer, following manufacturers' instructions. Infliximab (Remicade[®], Merck & Co., Inc., Whitehouse Station, NJ) or adalimumab (Humira[®], Abbott Immunology, Chicago, IL) or etanercept (Enbrel[®], Wyeth, Philadelphia, PA), each one at the concentration of 1 µg/ml, were incubated for 24 hours at 37°C with either active MMP-3 or active MMP-12 (0.001-1 µg/ml) or active MMP-9 (1 µg/ml) or p-APMA 0.02 mM in TCNB buffer or TCNB buffer alone. In parallel, infliximab or adalimumab or etanercept, each one at the concentration of 1 µg/ml, were incubated for 24 hours at 37°C with 1 µg/ml active MMP-3 or MMP-12 or p-APMA 0.2 mM in TCNB buffer or TCNB buffer alone, and the reaction was stopped at fixed time points (3, 6, 10 and 24 hours). In parallel, increasing concentrations (0.01-10 µg/ml) of infliximab or adalimumab or etanercept were co-incubated for 24 hours with p-APMA 0.02 mM in TCNB buffer or 1 µg/ml active MMP-3 or MMP-12. In parallel, etanercept 0.1 µg/ml was incubated for 24 hours at 37°C with 1 µg/ml active MMP-3 or MMP-12 or p-APMA 0.2 mM in TCNB buffer or TCNB buffer alone, in the absence or presence of increasing concentrations (0.001-1 µM) of the broad-spectrum MMP inhibitor marimastat (Sigma-Aldrich) or the selective MMP-3/MMP-12 inhibitor UK370106 (Santa Cruz Biotechnology, Dallas, TX). Moreover, to assess the effect of mucosal protein extracts on TNF neutralizing agents, infliximab, adalimumab and etanercept, all at the concentration of 1 µg/ml, were co-incubated for 24 hours with either PBS only or with mucosal protein extracts. In parallel, increasing concentrations (0.01-10 µg/ml) of infliximab or adalimumab or etanercept were co-incubated for 24h with either PBS only or with mucosal protein extracts. In parallel, infliximab, adalimumab and etanercept, all at the concentration of 0.1 µg/ml, were incubated for 24h at 37°C with either PBS only or with IBD mucosal protein extracts, in the absence or presence of increasing concentrations (0.001-1 µM) of marimastat (Sigma-Aldrich) or UK370106 (Santa Cruz Biotechnology). Cleavage reaction products were then stored at -70°C until further analysis.

5.2.6 Protein G binding assay

The effect of proteases on the Fc region of TNF- α neutralising agents was assessed by means of a protein G binding assay. Untreated or MMP-3-/MMP-12-treated TNF- α neutralising agents were added to the wells of a protein G-coated plate (Fisher Scientific UK). While human IgG can bind to protein G through their Fc region, chicken antibodies are unable to bind to protein G. Therefore, chicken anti-human p75 TNF receptor antibody (Abcam) which had been previously conjugated with horseradish peroxidase using a commercial kit (Abcam), or horseradish peroxidase-conjugated chicken anti-human F(ab')₂ (Fisher Scientific UK) were then added to the plate as a method to detect, respectively, the amount of etanercept or infliximab and adalimumab bound to the plate. Tetramethylbenzidine was then added and after stopping the reaction the optical density was measured (absorbance detection mode, 450 nm wavelength) on the Bio-tek Synergy™ HT Multi-Detection Microplate Reader (BioTek, Swindon, UK).

5.2.7 Luciferase assay

The effect of proteases on the TNF- α -neutralising function of anti-TNF- α agents was assessed by means of a HeLa cell line which had been stably transfected with the luciferase reporter gene under the control of NF- κ B enhancer elements (Kabouridis *et al.* 2002). HeLa cells were cultured for 6 hours in the absence or presence of 10 ng/ml rhTNF- α (R&D Systems), with or without 1 μ g/ml active MMP-3 or MMP-12 or increasing concentrations (0.01-10 μ g/ml) of infliximab or adalimumab or etanercept, all pre-treated for 24 hours with either p-APMA 0.02 mM in TCNB buffer or 1 μ g/ml active MMP-3 or MMP-12. In parallel, HeLa cells were cultured in the absence or presence of 10 ng/ml rhTNF- α , with 0.1 μ g/ml etanercept, pre-incubated for 24 hours with TCNB or 1 μ g/ml active MMP-3 or MMP-12, in the absence or presence of increasing concentrations (0.001-1 μ M) of marimastat (Sigma-Aldrich) or UK370106 (Santa Cruz Biotechnology). In

parallel, HeLa cells were cultured with 10 ng/ml rhTNF- α , with or without increasing concentrations (0.01-10 μ g/ml) of infliximab or adalimumab or etanercept, all pre-treated for 24 hours with either PBS or mucosal protein extracts prepared in PBS. In parallel, HeLa cells were cultured for 6 hours in the absence or presence of 10 ng/ml rhTNF- α , with or without 0.1 μ g/ml of infliximab, adalimumab or etanercept, all pre-treated for 24 hours with either PBS or mucosal proteins from IBD patients, in the absence or presence of increasing concentrations (0.001-1 μ M) of marimastat (Sigma-Aldrich) or UK370106 (Santa Cruz Biotechnology). The direct effect of IBD protein extracts and MMP inhibitors on the production of luciferase by HeLa cells was also assessed. HeLa cells were cultured for 6 hours in the absence or presence of 10 ng/ml rhTNF- α (R&D Systems), with or without IBD mucosal protein extracts prepared in PBS, in the absence or presence of 1 μ M marimastat (Sigma-Aldrich) or 1 μ M UK370106 (Santa Cruz Biotechnology). After culture, HeLa cells were lysated and assayed for luciferase activity with a commercial kit (Promega UK), following the manufacturers' instructions, using (luminescence detection mode, filters set in open position) the Bio-tek Synergy™ HT Multi-Detection Microplate Reader (BioTek).

5.2.8 Cleaved anti-TNF- α agent detection

The presence of clipped infliximab or adalimumab in human sera of IBD patients was investigated by a newly set-up ELISA assay. rhTNF- α (R&D Systems) 10 μ g/ml was added to Nunc Clear Maxisorp 96-well plates (Sigma-Aldrich). After 16 hour incubation at +4°C, plates were blocked for 30 minutes at 20-26°C in 3% bovine serum albumine (Sigma-Aldrich) in PBS. Three-fold serial dilutions of IBD sera, and of MMP-3- and MMP-12-cleaved infliximab (kindly provided by our collaborator R.J. Brezski – starting from 2 μ g/ml), which I used as standard, were added to the plates. After 1 hour at 20-26°C, 2.5 μ g/ml biotinylated rabbit anti-human MMP-3/MMP-12 IgG hinge cleavage site monoclonal antibody (kindly

provided by R.J. Brezski) were added. After 1 hour at 20-26°C, horseradish peroxidase-conjugated streptavidin (R&D Systems) 1:10000 was added to the plates. After 1 hour and thorough washing, tetramethylbenzidine was added and after stopping the reaction the optical density was measured (absorbance detection mode, 450 nm wavelength) on the Bio-tek Synergy™ HT Multi-Detection Microplate Reader (BioTek).

5.2.9 Cleaved endogenous IgG detection

The presence of clipped endogenous IgG in human sera of IBD patients and control subjects was studied by a newly set-up ELISA assay. Streptavidin (Sigma-Aldrich) 10 µg/ml was added to Nunc Clear Maxisorp 96-well plates (Sigma-Aldrich). After 16 hour incubation at +4°C, plates were blocked for 30 minutes at 20-26°C in 3% bovine serum albumine (Sigma-Aldrich) in PBS, then incubated for 1 hour at 20-26°C with 15 µg/ml biotinylated rabbit anti-human MMP-3/MMP-12 IgG hinge cleavage site monoclonal antibody (kindly provided by our collaborator R.J. Brezski, Biologics Research, Janssen Research & Development, Spring House, PA). Plates were then incubated for 1 hour at 20-26°C with three-fold serial dilutions of IBD and control sera, followed by an horseradish peroxidase-conjugated donkey anti-human IgG heavy and light chain antibody 1:10000 (Jackson ImmunoResearch, West Grove, PA), that has minimum cross-reactivity with rabbit IgG. After 1 hour and thorough washing, tetramethylbenzidine was added and after stopping the reaction the optical density was measured (absorbance detection mode, 450 nm wavelength) on the Bio-tek Synergy™ HT Multi-Detection Microplate Reader (BioTek).

5.2.10 Anti-hinge autoantibody detection

Finally, anti-hinge autoantibodies against the immunogenic neo-epitopes generated by the proteolytic cleavage of endogenous IgG in human sera of IBD patients and control subjects were measured by a newly set-up ELISA assay.

Streptavidin (Sigma-Aldrich) 10 µg/ml was added to Nunc Clear Maxisorp 96-well plates (Sigma-Aldrich). After 16 hour incubation at +4°C, plates were blocked for 30 minutes at 20-26°C in 3% bovine serum albumine (Sigma-Aldrich) in PBS, then incubated for 1 hour at 20-26°C with 1 µM biotinylated 14mer peptide analogue of the human IgG1 hinge with a C-terminal amino acid corresponding to the MMP-3/MMP-12 cleavage site (kindly provided by our collaborator R.J. Brezski). Plates were then incubated for 1 hour at 20-26°C with three-fold serial dilutions of a chimaeric rabbit/human anti-human MMP-3/MMP-12 IgG hinge cleavage site monoclonal antibody – which I used as a standard – or sera from 62 active IBD patients and from 20 control subjects, followed by an horseradish peroxidase-conjugated goat anti-human IgG Fc antibody 1:10000 (Jackson ImmunoResearch). After 1 hour and thorough washing, tetramethylbenzidine was added and after stopping the reaction the optical density was measured (absorbance detection mode, 450 nm wavelength) on the Bio-tek Synergy™ HT Multi-Detection Microplate Reader (BioTek). Similarly to a commercial ELISA, a concentration curve was constructed based on the optical densities recorded for the standard (which was added to the plate with two-fold serial dilutions starting from 10 ng/ml), and the corresponding equation (optical density = $0.5143 \times \text{concentration} + 0.1344$) allowed to convert serum anti-hinge autoantibody optical density into concentration (ng/ml).

5.3 Results

5.3.1 Effect of MMP-3, MMP-12 and MMP-9 on the integrity of anti-TNF- α agents

Increasing concentrations (0.001-1 $\mu\text{g/ml}$) of MMP-3 or MMP-12 were co-incubated for 24 hours with 1 $\mu\text{g/ml}$ (therapeutic serum levels in patients) (Tracey *et al.* 2008) infliximab, adalimumab and etanercept, and cleavage patterns were analysed in reducing gels by immunoblotting using an anti-human IgG Fc antibody (Fig. 5.1). Infliximab, adalimumab and etanercept were clipped in a concentration-dependent manner by both MMP-3 and MMP-12, with the formation of a 32 kDa fragment, which likely corresponds to the Fc monomer observed after MMP digestion of native human IgG₁ (Brezski *et al.* 2009) (Fig. 5.1A). An additional ~50 kDa etanercept fragment appeared at low MMP concentrations, which was not predicted based on the known MMP-3/MMP-12 cleavage site on the lower hinge of human IgG₁. Densitometry of the percentage of intact anti-TNF- α agents remaining at each MMP concentration showed that etanercept is markedly more susceptible to clipping than infliximab and adalimumab (Fig. 5.1B). In parallel, 1 $\mu\text{g/ml}$ MMP-9 was co-incubated for 24 hours with 1 $\mu\text{g/ml}$ infliximab, adalimumab and etanercept, and cleavage patterns were analysed in non-reducing gels by immunoblotting using an anti-human IgG Fc antibody (Fig. 5.2). Infliximab, adalimumab and etanercept were not cleaved by MMP-9.

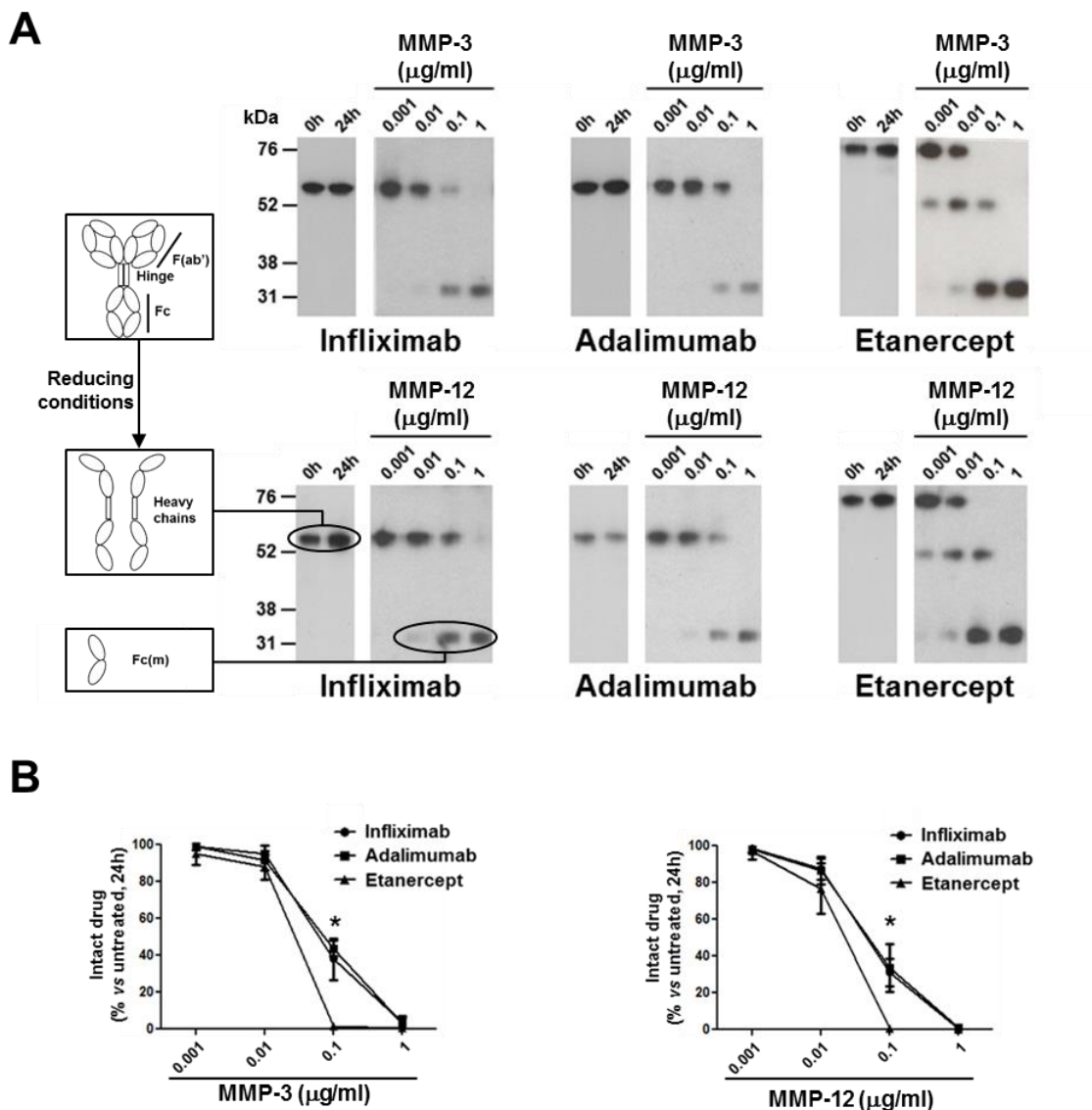


Figure 5.1

Figure 5.1. Degradation of anti-TNF- α agents by MMP-3 and MMP-12. (A) Infliximab, adalimumab and etanercept (1 $\mu\text{g/ml}$) were co-incubated at 37°C for 24 hours with increasing concentrations (0.001-1 $\mu\text{g/ml}$) of p-APMA-activated rhMMP-3 or rhMMP-12, and were visualised by immunoblotting in reducing conditions using an anti-human IgG Fc primary antibody. The heavy chains of infliximab and adalimumab appeared as a single band of ~55 kDa and the monomers of etanercept appeared as a single band of 75 kDa, and remained intact after 24 hour co-incubation with or without p-APMA alone (0h, 24h on the left of each blot). All TNF- α neutralising agents were cleaved by MMP-3 or MMP-12 with the formation of a 32 kDa band, likely to correspond to the Fc monomer observed after MMP digestion of native human IgG₁. Cleavage occurred in a concentration-dependent manner, as shown by the increasing intensity of the 32

kDa band at higher MMP concentrations and the decreasing intensity of the bands correspondent to the intact heavy chain of infliximab and adalimumab and to the intact etanercept monomer. At the lowest MMP concentrations, another ~50 kDa etanercept fragment appeared. Blots are representative of six separate experiments. **(B)** Densitometric analysis of the intact infliximab and adalimumab heavy chain, and of the intact etanercept monomer, after co-incubation with increasing concentrations of activated rhMMP-3 or rhMMP-12. Etanercept was more susceptible to MMP degradation than infliximab and adalimumab, and the band corresponding to the intact etanercept monomer totally disappeared after co-incubation with 0.1 $\mu\text{g/ml}$ MMP-3 and MMP-12. Results are expressed as mean percentage of the intact anti-TNF- α agent after MMP treatment compared to the correspondent untreated TNF- α neutralising agent after 24 hour co-incubation with or without p-APMA \pm s.d. (n=6). Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test. * $p < 0.001$ versus MMP-3-/MMP-12-treated etanercept at the correspondent MMP concentration.

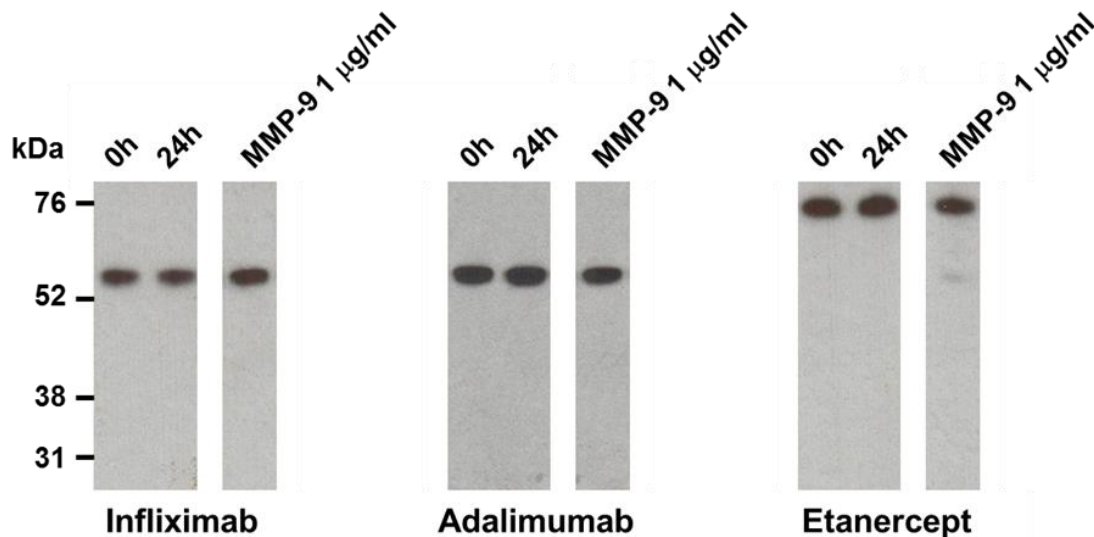


Figure 5.2

Figure 5.2. Effect of MMP-9 on the integrity of anti-TNF- α agents. Infliximab, adalimumab and etanercept (1 $\mu\text{g/ml}$) were co-incubated at 37°C for 24 hours with 1 $\mu\text{g/ml}$ activated rhMMP-9, and were visualised by immunoblotting in reducing conditions using an anti-human IgG Fc primary antibody. Infliximab, adalimumab and etanercept were not cleaved by MMP-9. Blots are representative of six separate experiments.

5.3.2 Sequential cleavage of anti-TNF- α agents by MMP-3 and MMP-12

Each anti-TNF- α agent (1 μ g/ml) was also co-incubated with a fixed concentration (1 μ g/ml) of MMP-3 or MMP-12. Subsequent immunoblotting in non-reducing gels with anti-human Ig κ light chain showed that degradation of infliximab and adalimumab occurs in a time-dependent sequential manner, with the formation of single-cleaved – upon removal of one Fc monomer – and, subsequently, double-cleaved – F(ab')₂ – intermediate products (Figure 5.3). Etanercept could not be visualised using anti-human Ig κ primary antibody.

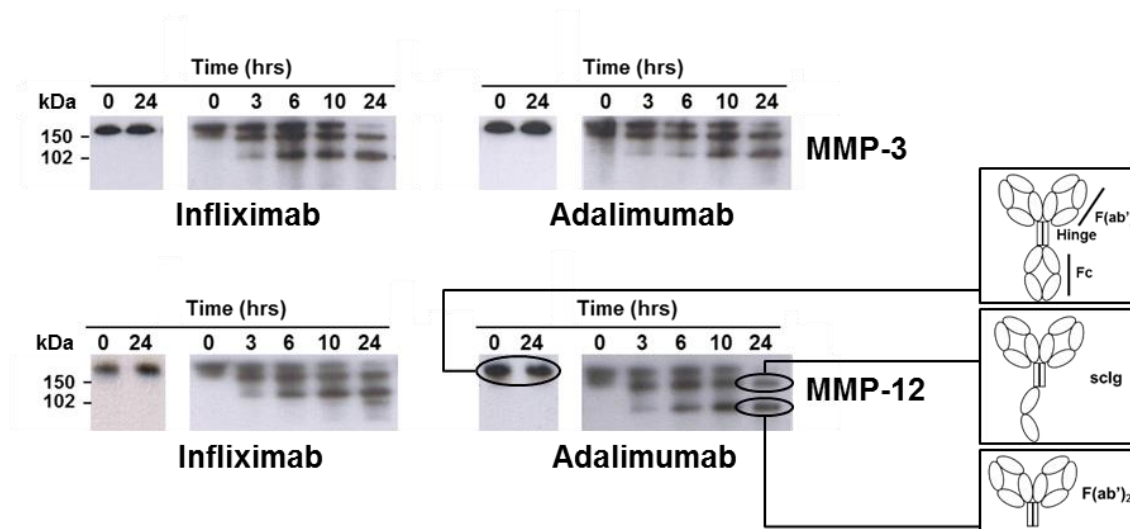


Figure 5.3

Figure 5.3 Sequential cleavage of infliximab and adalimumab by MMP-3 and MMP-12. Infliximab and adalimumab (1 μ g/ml) were co-incubated at 37°C with 1 μ g/ml activated rhMMP-3 or rhMMP-12. The incubation was interrupted at fixed time points (3, 6, 10 and 24 hours), and the anti-TNF- α agents were visualised by immunoblotting in non-reducing conditions using an anti-human Ig κ light chain antibody. Infliximab and adalimumab were cleaved in a time-dependent manner by either MMP-3 or MMP-12 with the sequential formation of two additional ~130 and ~100 kDa bands, which likely correspond to single-cleaved Ig intermediate (scIg) and F(ab')₂ fragments, respectively, and increased in intensity over time. Of note, the bands corresponding to intact infliximab and adalimumab decreased in intensity over time.

5.3.3 Effect of MMP-3 and MMP-12 on protein G binding capacity of anti-TNF- α agents

To confirm that MMP-3 and MMP-12 had indeed removed the Fc region, an ELISA using protein G-coated plates was performed on samples of TNF- α neutralising agents pre-incubated with increasing concentrations of activated MMP-3 and MMP-12, using chicken secondary antibodies against F(ab')₂ or p75 TNFR (Fig. 5.4). In this experiment, only intact anti-TNF- α agents can bind to the protein G-coated plate, are then recognised by secondary antibodies and give a positive signal, therefore the optical density can be considered as an indication of the amount of residual intact anti-TNF- α agent. Untreated TNF- α neutralising agents bound strongly to protein G, but after 24 hour of incubation with MMP-3 or MMP-12, binding decreased in a dose-dependent fashion. The kinetics of the loss of protein G binding were faster for etanercept than for the other agents tested. After pre-incubation with 0.1 μ g/ml of either MMP-3 or MMP-12, there was still substantial binding with infliximab and adalimumab, but etanercept binding was minimal.

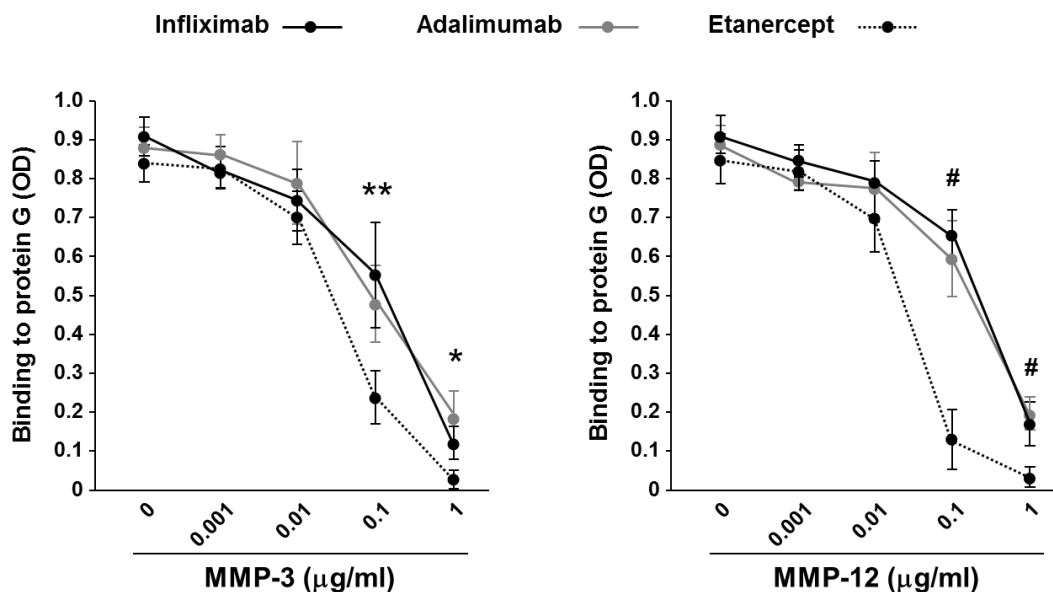


Figure 5.4

Figure 5.4 (see previous page). **Effect of MMP-3 and MMP-12 on protein G binding capacity of anti-TNF- α agents.** TNF- α neutralising agents, all at the concentration of 1 μ g/ml, were co-incubated for 24 hours with increasing concentrations (0.001-1 μ g/ml) of active rhMMP-3 or rhMMP-12. Untreated or MMP-3- and MMP-12-treated TNF- α neutralising agents were added to the wells of protein G-coated plates. Horseradish peroxidase-conjugated chicken anti-human F(ab')₂ or anti-human TNF receptor II antibodies, which do not bind to protein G, were used to detect and quantify protein G binding to TNF- α neutralising agents. Values, expressed as optical density (OD), are mean \pm s.d. of six separate experiments. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test. * $p < 0.05$ *versus* etanercept at the correspondent MMP concentration; ** $p < 0.01$ *versus* etanercept at the correspondent MMP concentration; # $p < 0.001$ *versus* etanercept at the correspondent MMP concentration.

5.3.4 Effect of MMP-3 and MMP-12 on TNF- α neutralisation ability of anti-TNF- α agents

A nuclear factor- κ B reporter cell line was then used to investigate the effect of MMP-3 and MMP-12 on the ability of each biologic agent to neutralise soluble TNF- α (Fig. 5.5). In the absence of MMPs, infliximab, adalimumab and etanercept effectively inhibited TNF- α -induced luciferase activity. Neutralisation of TNF- α by infliximab and adalimumab did not change after overnight incubation with MMP-3/MMP-12, whereas etanercept showed a significant reduction in its ability to neutralise TNF- α . Collectively, these results show that the removal of the Fc region by MMP-3 and MMP-12 does not affect the ability of infliximab and adalimumab to neutralise soluble TNF- α as F(ab')₂ fragments, even at the lowest anti-TNF/MMP concentration ratio of 1:10, but does compromise significantly the TNF- α neutralising function of etanercept.

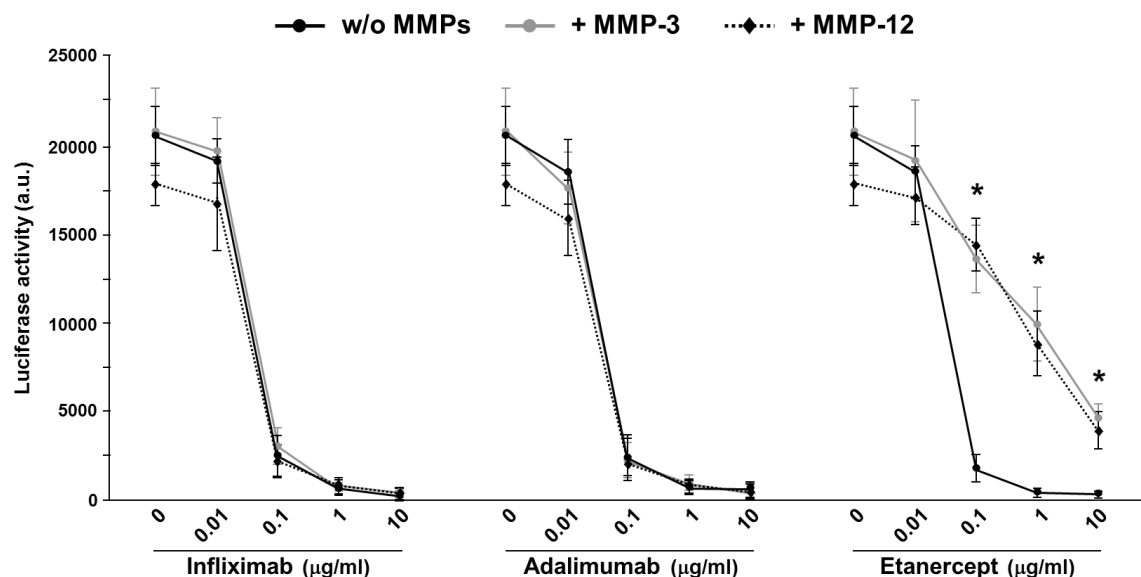


Figure 5.5

Figure 5.5. Effect of MMP-3 and MMP-12 on TNF- α neutralising function of anti-TNF- α agents. NF- κ B reporter HeLa cells were cultured for 6 hours in the absence or presence of 10 ng/ml rhTNF- α , with increasing concentrations (0.01-10 μ g/ml) of infliximab, adalimumab or etanercept, all pre-treated for 24 hours with p-APMA; w/o MMPs) or 1 μ g/ml active MMP-3 or MMP-12. After culture, HeLa cell lysates were assayed for luciferase activity. Values, expressed as arbitrary units (a.u.), are means \pm s.d. of six separate experiments. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test. * $p < 0.001$ versus TNF- α plus p-APMA-treated etanercept at the correspondent concentration.

5.3.5 Effect of MMP-3 and MMP-12 inhibition on TNF- α neutralisation ability of MMP-exposed etanercept

Subsequently, the ability of the broad-spectrum MMP inhibitor marimastat (which however has no inhibitory effect on MMP-12) or the selective MMP-3/MMP-12 inhibitor UK370106 to prevent the loss of function of MMP-exposed etanercept was investigated (Fig. 5.6). In particular, based on my results showing maximal loss of TNF- α neutralising ability when 0.1 μ g/ml etanercept was pre-incubated with 1 μ g/ml MMPs, I tested the effect of 1 μ g/ml active rhMMP-3 or MMP-12 on

0.1 $\mu\text{g/ml}$ etanercept in the absence or presence of increasing concentrations (0.001-1 μM) of marimastat or UK370106. The addition of UK370106 to the cleavage reactions with either MMP-3 or MMP-12 effectively restored TNF- α neutralising function of etanercept in a dose-dependent manner. Conversely, marimastat was able to prevent only MMP-3- but not MMP-12-induced loss of TNF- α neutralising ability of etanercept. This shows that the loss of TNF- α neutralising function of etanercept induced by MMP-3 and MMP-12 can be specifically prevented by the MMP inhibitors.

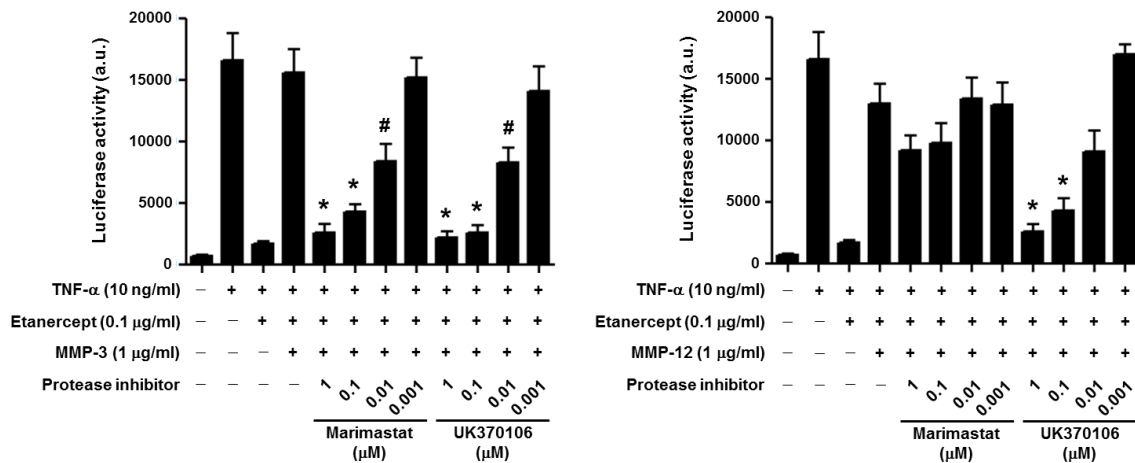


Figure 5.6

Figure 5.6. Effect of MMP inhibitors on TNF- α neutralising function of MMP-exposed etanercept. Nuclear factor- κB reporter HeLa cells were cultured for 6 hours in the absence or presence of 10 ng/ml rhTNF- α , with 0.1 $\mu\text{g/ml}$ etanercept, pre-incubated for 24 hours with 1 $\mu\text{g/ml}$ active rhMMP-3 or rhMMP-12 in the absence or presence of increasing concentrations (0.001-1 μM) of marimastat or UK370106. After culture, HeLa cell lysates were assayed for luciferase activity. Values, expressed as arbitrary units (a.u.), are means \pm s.d. of six separate experiments. Data were analysed by ANOVA One way analysis of variance followed by the Dunnett's multiple comparison test. # $p < 0.01$ versus TNF- α plus MMP-treated etanercept; * $p < 0.001$ versus TNF- α plus MMP-treated etanercept.

5.3.6 MMP-3/MMP-12 activity in IBD and control protein extracts

Subsequently, protein extracts from normal colonic biopsies of control subjects and from inflamed colonic biopsies of IBD patients were prepared, and differences in the amounts of tissue-extracted proteins from the different patient samples were controlled by measuring total protein concentration, then normalising it to 2 mg/ml by dilution in PBS. MMP-3/MMP-12 activity was then measured by a FRET assay, using rhMMP-3 and rhMMP-12 as positive controls (Fig. 5.7). IBD protein extracts showed a significantly higher MMP-3 and MMP-12 activity compared to control protein extracts. No difference between CD and UC protein extracts was observed. Interestingly, MMP-3/MMP-12 activity in IBD protein extracts was significantly lower than rhMMP-3 and rhMMP-12, each one at the concentration of 1 μ g/ml.

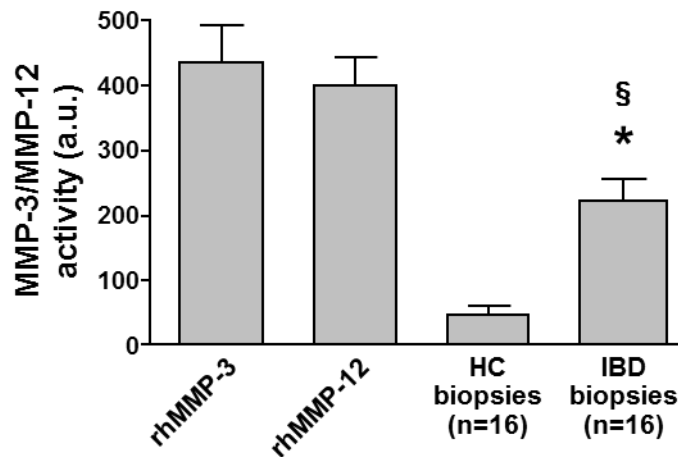


Figure 5.7

Figure 5.7. MMP-3/MMP-12 activity in IBD and control protein extracts. MMP-3/MMP-12 activity in the protein extracts of normal colonic biopsies of eight control subjects (HC) and in inflamed colonic biopsies of 16 IBD patients. Active rhMMP-3 and rhMMP-12 have been used as positive controls. Bars, expressed as arbitrary units (a.u.), are means \pm s.d. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test. * $p < 0.01$ versus HC biopsies. § $p < 0.05$ versus rhMMP-3 and rhMMP-12.

5.3.7 Effect of mucosal protein extracts on the integrity of anti-TNF- α agents

Subsequently, the protein extracts from control subjects and inflamed IBD colonic mucosa were co-incubated with TNF- α neutralising agents, and cleavage patterns were analysed in reducing gels by immunoblotting using an anti-human IgG Fc antibody (Fig. 5.8). No cleavage by control protein extracts was detected, whereas infliximab, adalimumab and etanercept were cleaved by IBD proteins, suggesting that the increased proteolytic activity present in chronically inflamed tissues may affect drug stability. While infliximab and adalimumab were partially cleaved, intact etanercept was completely lost after co-incubation with IBD protein extracts. In some cases the 32 kDa fragment, indicative of lower hinge cleavage by MMP-3/MMP-12 (Fig. 5.8 – patient #2), could be observed, nevertheless the heterogeneous clipping profile between different patients suggests that the tissue proteolytic profile varies among individuals and other proteases may also contribute to IgG cleavage.

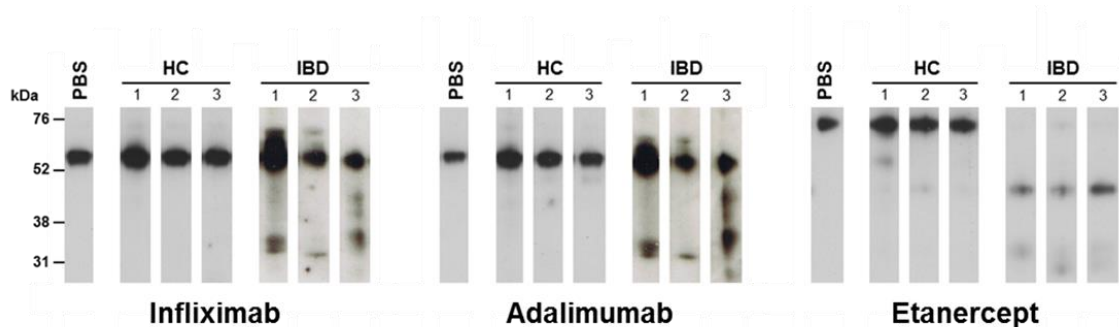


Figure 5.8

Figure 5.8. Effect of IBD mucosal protein extracts on anti-TNF- α agent integrity. Infliximab, adalimumab and etanercept, all at the concentration of 1 μ g/ml, were co-incubated for 24 hours with PBS only or with protein extracts from the colon of control subjects (HC) or from the colon of IBD patients. Infliximab, adalimumab and etanercept were visualised by immunoblotting in reducing conditions using an anti-human IgG Fc primary antibody. The heavy chains of infliximab and adalimumab appeared as a single band of ~55 kDa, remained intact after 24 hour co-incubation with PBS or HC protein extracts, and were partially cleaved by IBD mucosal proteins with the appearance of 32, ~35 and

~40 kDa fragments. Etanercept, which was minimally cleaved by HC mucosal proteins, was completely cleaved by inflamed IBD mucosal proteins with the formation of 32, ~35 and ~40, ~45 and ~50 kDa fragments. The 32 kDa band is consistent with lower hinge cleavage. Blots are representative of experiments performed with colonic mucosal proteins from eight HC subjects and eight IBD patients.

5.3.8 Effect of IBD mucosal protein extracts on TNF- α neutralisation ability of anti-TNF- α agents

Additionally, the effect of tissue extracts from normal and IBD colonic mucosa on the ability of each agent to neutralise soluble TNF- α was investigated (Fig. 5.9). The ability to neutralise TNF- α by the three agents was not altered after 24 hour co-incubation with proteins from control colonic mucosa. At low concentration (0.1 μ g/ml), infliximab and adalimumab showed a reduction in their ability to neutralise soluble TNF- α after co-incubation with IBD mucosal tissue extracts. Etanercept significantly lost its ability to neutralise TNF- α after co-incubation with proteins from inflamed IBD mucosa.

Figure 5.9 (see next page). **Effect of mucosal protein extracts on TNF- α neutralisation capacity of anti-TNF- α agents.** NF- κ B reporter HeLa cells were cultured for 6 hours in the absence or presence of 10 ng/ml rhTNF- α , with or without increasing concentrations (0.01-10 μ g/ml) of infliximab, adalimumab or etanercept, all pre-treated for 24 hours with either PBS or mucosal proteins from eight control subjects (HC) or eight IBD patients. After culture, HeLa cell lysates were assayed for luciferase activity. Values, expressed as arbitrary units (a.u.), are means \pm s.d. of eight separate experiments. Data were analysed by ANOVA One way analysis of variance followed by the Tukey's multiple comparison test. * $p < 0.001$ *versus* TNF- α plus PBS/HC-treated infliximab or adalimumab or etanercept at the correspondent concentration.

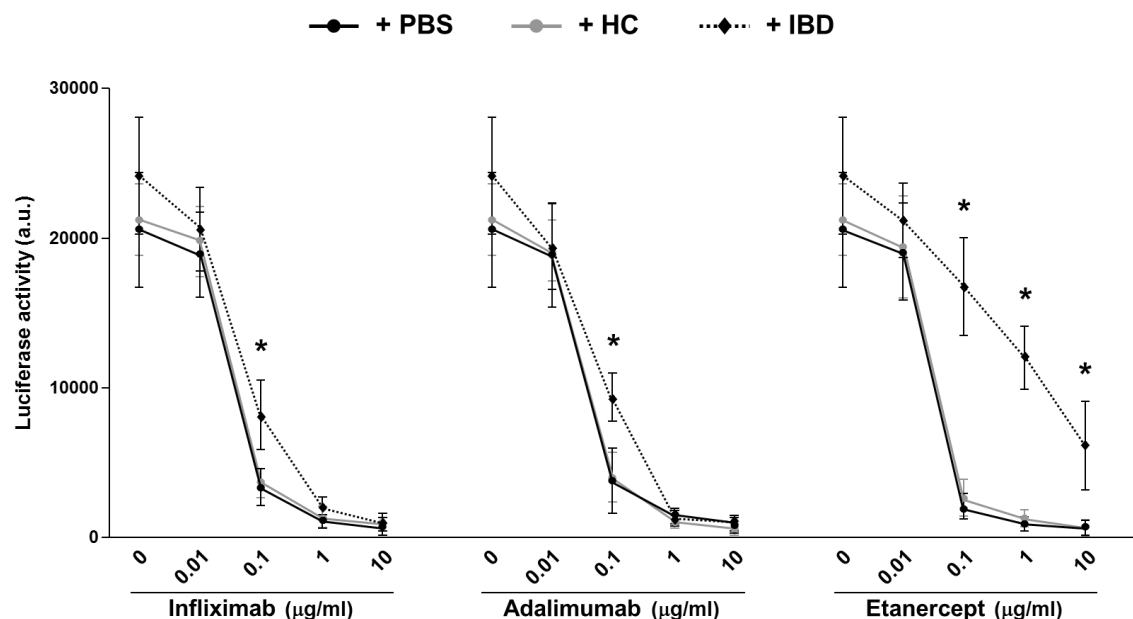


Figure 5.9

5.3.9 Effect of MMP-3 and MMP-12 inhibition on TNF- α neutralisation ability of anti-TNF- α agents exposed to IBD protein extracts

The ability of marimastat or the selective MMP-3/MMP-12 inhibitor UK370106 to prevent the loss of function of anti-TNF- α agents exposed to IBD mucosal protein extracts was then assessed (Fig. 5.10). In particular, based on my results showing maximal loss of TNF- α neutralising ability when 0.1 μ g/ml etanercept was pre-incubated with IBD mucosal protein extracts, I tested the effect of IBD tissue extracts on 0.1 μ g/ml of each anti-TNF- α agent in the absence or presence of increasing concentrations (0.001-1 μ M) of marimastat or UK370106. Marimastat, but not UK370106, was able to restore the ability of infliximab and adalimumab to neutralise soluble TNF- α upon exposure to IBD tissue extracts. Both marimastat and, to a lesser extent, UK370106 were able to restore the ability of etanercept to neutralise soluble TNF- α upon exposure to IBD tissue extracts. These findings suggest that, in addition to MMP-3 and MMP-12, other proteolytic enzymes may contribute significantly to the loss of TNF- α neutralising

function of anti-TNF- α agents exposed to IBD mucosal protein extracts.

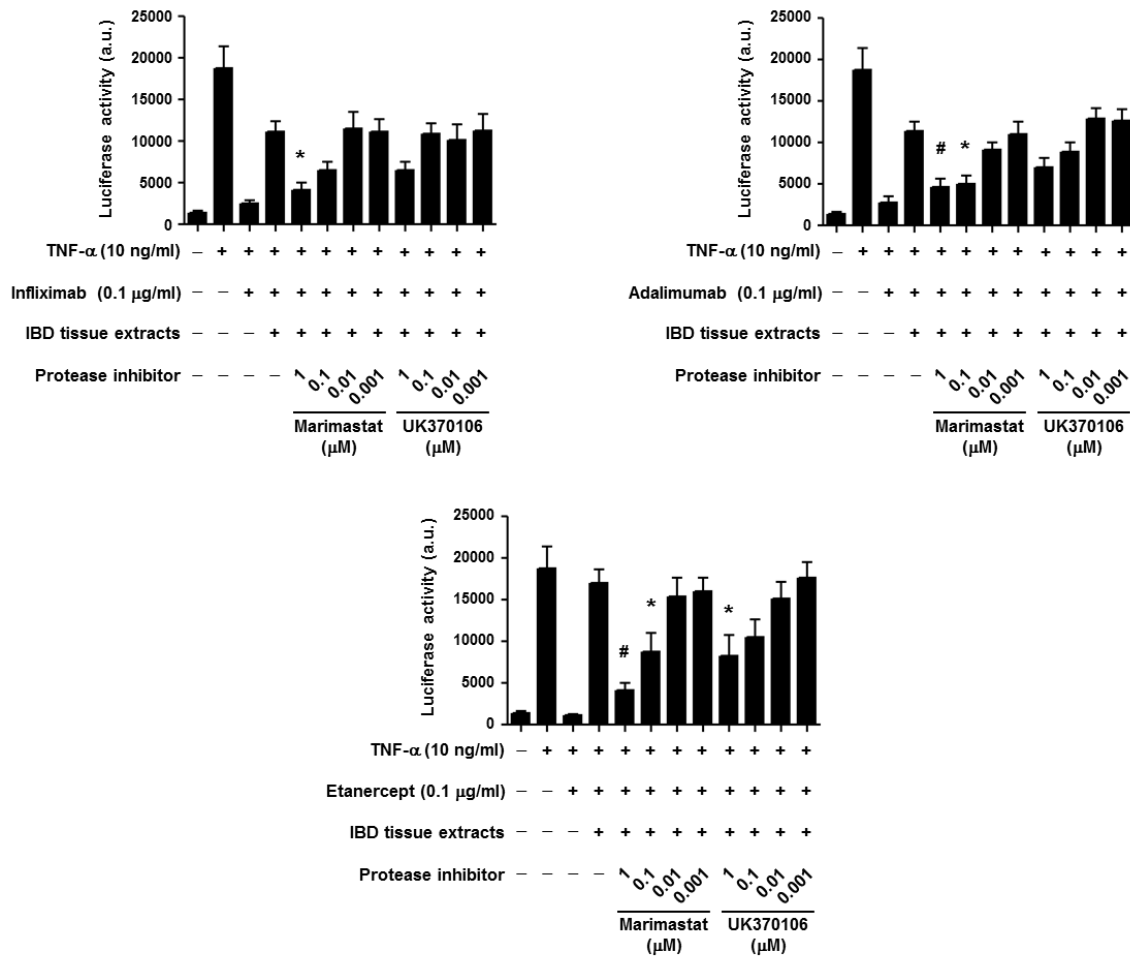


Figure 5.10

Figure 5.10. Effect of MMP inhibitors on TNF- α neutralising function of anti-TNF- α agents exposed to IBD protein extracts. Nuclear factor- κ B reporter HeLa cells were cultured for 6 hours in the absence or presence of 10 ng/ml rhTNF- α , with 0.1 μ g/ml infliximab, adalimumab or etanercept, all pre-treated for 24h with either PBS or mucosal tissue extracts from eight IBD patients in the absence or presence of increasing concentrations (0.001-1 μ M) of marimastat or UK370106. After culture, HeLa cell lysates were assayed for luciferase activity. Values, expressed as arbitrary units (a.u.), are means \pm s.d. of eight separate experiments. Data were analysed by ANOVA One way analysis of variance followed by the Dunnett's multiple comparison test. * p <0.05 versus TNF- α plus the corresponding IBD tissue extract-treated anti-TNF- α agent; # p <0.001 versus TNF- α plus the corresponding IBD tissue extract-treated anti-TNF- α agent.

5.3.10 Detection of MMP-3-/MMP-12-cleaved anti-TNF- α agents in IBD sera

In order to measure the concentration of clipped anti-TNF- α agents in the serum of IBD patients, sera collected at the time of cannulation a few minutes before the following infliximab infusion were collected (i.e. at least two weeks after the previous one). Subsequently, an ELISA assay was developed in which sera were incubated on a TNF- α -coated plate, then a biotinylated antibody specific for the MMP-3 and MMP-12 cleavage sites on the hinge region of human IgG was used to reveal the presence of any clipped infliximab in the serum. Clipped infliximab was undetectable (data not shown), and this could be due to a number of reasons. The first could be that clipped anti-TNF- α agents may not have the same affinity as intact anti-TNF- α agents for TNF- α absorbed on the plate. One further explanation may be the catabolism of degraded IgG in the samples collected immediately before the following administration.

5.3.11 Detection of MMP-3-/MMP-12-cleaved endogenous IgG in IBD and control sera

In order to test whether proteolytic cleavage occurs *in vivo* and has clinical relevance in IBD patients, we hypothesised that we could detect clipped endogenous IgG in patients' sera. Indeed, the ELISA assay which was performed by absorbing on the plate the biotinylated antibody specific for the MMP-3 and MMP-12 cleavage sites on the hinge region of human IgG revealed that sera from active IBD patients contained significantly higher levels of MMP-3-/MMP-12-cleaved endogenous IgG compared to control subjects (Fig. 5.11A). IBD patients were then stratified according to their subsequent response to biologic therapy, and, among patients who did not respond to infliximab or adalimumab therapy, a subset of patients had significantly higher serum levels of MMP-3-/12-cleaved IgG than responders (Fig. 5.11B).

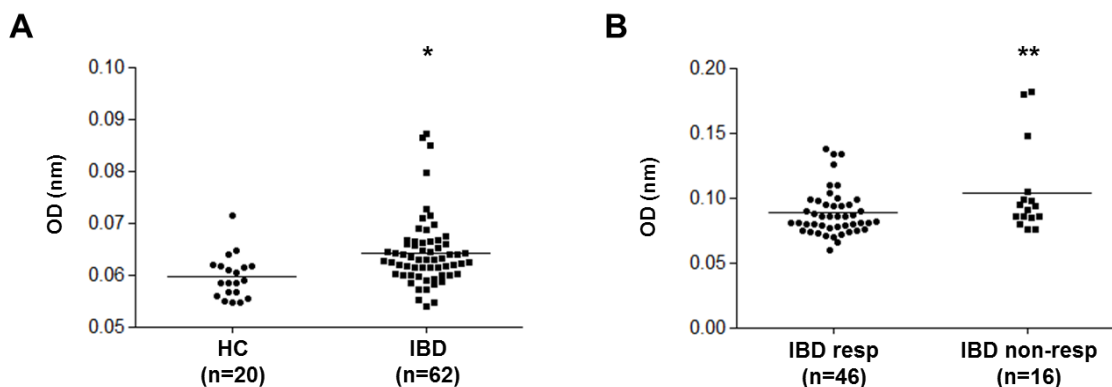


Figure 5.11

Figure 5.11. Levels of MMP-3/MMP-12-cleaved endogenous IgG in IBD and control sera. (A) ELISA plates were coated with streptavidin, followed by a biotinylated rabbit anti-human MMP-3/MMP-12 IgG hinge cleavage site monoclonal antibody. Sera from 62 active IBD patients who were going to receive the first infliximab infusion, and from 20 control subjects (HC) were serially diluted and added to the plate, then a horseradish peroxidase-conjugated donkey anti-human IgG heavy and light chain antibody was used to detect bound cleaved IgG. Levels of clipped endogenous IgG are expressed as optical densities (OD). The horizontal bar indicates the mean OD. Data shown here refer to the highest serum dilution (1:12150) at which I could detect significantly different signals between HC and IBD sera. (B) Levels of clipped endogenous IgG in the serum of IBD patients were stratified as IBD responder patients (IBD resp; n=46) and IBD non-resp (n=16; 6 CD and 10 UC patients) according to their subsequent response to infliximab treatment. No difference in disease severity and/or CRP serum levels were present at baseline between IBD resp and IBD non-resp patients. Levels are expressed as OD. The horizontal bar indicates the mean OD. Data shown here were obtained at a 1:1350 dilution of the sera, which is the highest serum dilution at which I could detect significantly different signals between IBD resp and IBD non-resp sera. The unpaired two-tailed *t* test was applied to compare between HC and IBD sera, and between IBD resp and IBD non-resp sera. **p*<0.005 versus HC sera. ***p*<0.05 versus IBD resp sera.

5.3.12 Detection of anti-hinge autoantibodies in IBD and control sera

The IgG F(ab')₂ fragments generated as a result of proteolytic cleavage become self-antigens that lead to the formation of autoantibodies in the human population (Brezski *et al.* 2008), and serum anti-hinge autoantibodies are more frequent in

patients with rheumatoid arthritis compared to control subjects (Rispen *et al.* 2012). An additional cause of the inability to detect clipped anti-TNF- α agents could be the presence of anti-hinge autoantibodies bound to the drug, which may render the epitope inaccessible to the detection antibody. Therefore, an ELISA assay was developed and tested on the same IBD patient and control sera by coating the plate with the peptide sequence on the IgG hinge region generated by MMP-3/MMP-12 cleavage (Fig. 5.12). Sera from active IBD patients contained significantly higher concentrations of anti-hinge autoantibodies compared to control subjects (Fig. 5.12A). Moreover, among patients who subsequently did not respond to infliximab therapy, a subset of patients had significantly higher serum levels of anti-hinge autoantibodies compared to responders (Fig. 5.12B).

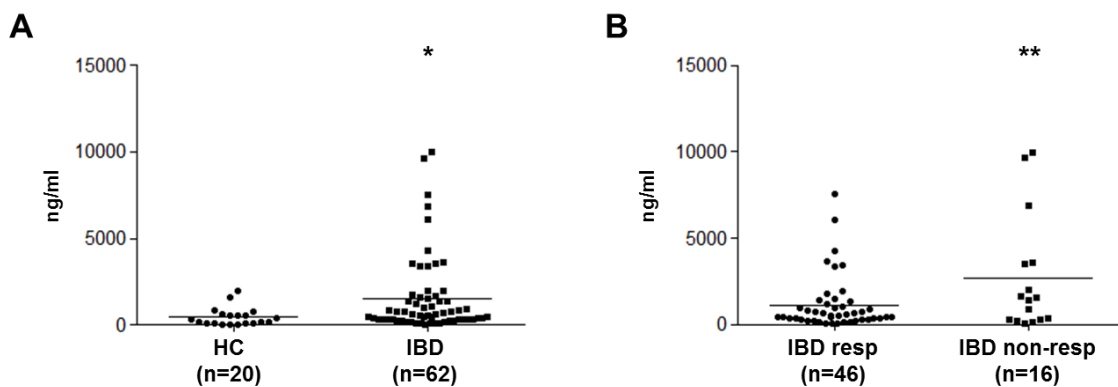


Figure 5.12

Figure 5.12. Levels of anti-hinge autoantibodies in IBD and control sera. (A) ELISA plates were coated with streptavidin, followed by the biotinylated 14mer peptide analogue of the human IgG₁ hinge with a C-terminal amino acid corresponding to the MMP-3/MMP-12 cleavage site. A chimaeric rabbit/human anti-human MMP-3/MMP-12 IgG hinge cleavage site monoclonal antibody, used as a standard, or sera from 62 active IBD patients who were going to receive the first infliximab infusion, and from 20 control subjects (HC) were serially diluted and added to the plate, then a horseradish peroxidase-conjugated goat anti-human IgG Fc antibody was used as detection antibody. A concentration curve was constructed based on the OD recorded for the standard, and the corresponding equation allowed to convert serum anti-hinge autoantibody OD into ng/ml. The horizontal bar indicates the mean concentration. (B) Levels of anti-hinge autoantibodies in the serum of IBD patients were stratified as IBD resp

(n=46) and IBD non-resp (n=16; 6 CD and 10 UC patients) according to their response to infliximab treatment. The horizontal bar indicates the mean concentration. Data shown here were obtained at a 1:1350 dilution of the sera, which is the highest serum dilution at which I could detect significantly different signals between HC and IBD sera, and between IBD resp and IBD non-resp sera. The unpaired two-tailed *t* test was applied to compare between HC and IBD sera, and between IBD resp and IBD non-resp sera. **p*<0.05 *versus* HC sera. ***p*<0.05 *versus* IBD resp sera.

5.4 Results summary

In the current study, we found that:

8. MMP-3 and MMP-12, but not MMP-9, cleave infliximab, adalimumab and etanercept *in vitro* in a dose-dependent manner. As a result of MMP degradation, only etanercept loses its TNF- α neutralisation ability.
9. None of the anti-TNF- α agents studied are degraded by proteins from control colonic mucosa. Protein extracts from inflamed IBD colonic mucosa impair the integrity of infliximab, adalimumab, and etanercept. At low concentration, infliximab, adalimumab, and etanercept lose TNF- α neutralisation ability after co-incubation with protein extracts from inflamed IBD colonic mucosa.
10. We were unable to detect MMP-3-/MMP-12-cleaved infliximab in IBD sera.
11. Overall, sera from active IBD patients and from IBD patients who subsequently do not respond to biologic therapy contain significantly higher levels of MMP-3-/MMP-12-cleaved endogenous IgG and anti-hinge autoantibodies compared to control subjects and to IBD patients who subsequently respond to biologic therapy. It needs to be noted, however, that there is a high degree of overlap between the groups considered.

5.5 Discussion

Our findings indicate that MMP-3, MMP-12, and protein extracts from inflamed IBD mucosa degrade infliximab, adalimumab and etanercept *in vitro*, however etanercept shows a higher susceptibility than infliximab and adalimumab. Furthermore, sera from active IBD patients contained higher levels of MMP-3-/MMP-12-cleaved endogenous IgG and anti-hinge autoantibodies compared to control subjects. This Section focuses on the interpretation of our findings in the context of the existing literature, on the limitations of our study, and on possible future experiments aimed at clarifying the relevance of MMP degradation as a mechanism of primary non-responsiveness to biologic therapy in IBD.

Effects of MMPs and IBD mucosal protein extracts on anti-TNF- α agents

Anti-TNF- α agents, despite being a powerful tool in the management of IBD, are burdened with high costs and relevant risks, and a significant percentage of patients do not respond to treatment. Non-responsiveness can be defined as primary, when patients do not show any improvement after the treatment start, or secondary, when patients initially improve and subsequently lose response to treatment (Komaki *et al.* 2016).

Multiple factors contribute to non-responsiveness to anti-TNF- α agents in IBD. One important factor may be represented by the high degree of disease heterogeneity present in IBD. Insufficient dosing may represent an alternative to disease heterogeneity in explaining primary non-responsiveness. As an example, a high body mass index may influence responsiveness to adalimumab, which, unlike infliximab, is administered at a fixed dose rather than based on the patient's weight (Travis *et al.* 2012a). The formation of anti-drug antibodies, especially against infliximab, exerts a profound influence on the persistence of functional TNF- α neutralising agents in the circulation and, more importantly, in the inflamed mucosa (Nanda *et al.* 2013). However, anti-drug antibodies are

important in the late induction phase, and are unlikely to play a role in primary non-responsiveness. The application of treatment algorithms based on serum trough levels of anti-TNF- α agents, and on the presence or absence of anti-drug antibodies, has shown promising results in IBD (Afif *et al.* 2010; Vande Casteele *et al.* 2014).

One of the mechanisms mediating the pro-inflammatory effects of T cell-derived cytokines in the inflamed IBD mucosa is represented by the overexpression of proteolytic enzymes, which can degrade ECM components and promote the formation of intestinal ulcers (Ravi *et al.* 2007). Proteolytic enzymes cleave human IgG₁ at different points on the amino acid sequence. In particular, proteases that cleave IgG₁ in the lower hinge region include MMP-3 and MMP-12; MMP-7, that has the same cleavage site as pepsin; and cathepsin G. As a result of the exposure to these proteases, F(ab')₂ fragments are released. Conversely, neutrophil elastase cleaves IgG₁ in the upper hinge region, between the plasmin and the papain cleavage sites, thereby generating single F(ab') fragments (Brezski *et al.* 2010). After MMP-3, MMP-12 and MMP-7 cleavage, a 32 kDa single-chain fragment is released, which is part of the Fc portion (Brezski *et al.* 2009). TNF- α neutralising biologic agents have at least part of their amino acid sequence in common with IgG₁ (Rutgeerts *et al.* 2009).

We observed that MMP-3 and MMP-12, but not MMP-9, cleave infliximab, adalimumab and etanercept *in vitro* in a dose-dependent, time-dependent, and sequential manner. MMP-3 and MMP-12 are capable of cleaving human IgG₁ exactly at the same point in the hinge region (Brezski *et al.* 2010). This explains why the analysis of anti-TNF- α integrity after digestion with these two MMPs displays very similar profiles. IBD mucosal protein extracts, which showed up-regulated MMP-3/MMP-12 activity compared to control mucosal protein extracts, were able to cleave infliximab and adalimumab. However, the degradation profile was heterogeneous, with evidence of several different fragments, suggesting that

various proteolytic enzymes present in IBD mucosa may degrade IgG₁ also in other points on the amino acid sequence than in the hinge region.

Anti-TNF- α agents may exert their therapeutic action via multiple mechanisms, including induction of apoptosis of activated immune cells, antibody-dependent cell-mediated cytotoxicity, and complement activation (Nesbitt *et al.* 2007). Similarly to what had been demonstrated for the monoclonal antibodies trastuzumab and rituximab (Clynes *et al.* 2000), the presence of the Fc region is necessary for antibody-dependent cell-mediated cytotoxicity and complement activation. A genetic polymorphism in IgG Fc γ receptor (Fc γ R)IIIa that enhances its affinity for IgG₁ is associated to increased biological response to infliximab in CD (Louis *et al.* 2004). Moreover, Vos *et al.* (Vos *et al.* 2011) have shown that the Fc region is required for the ability of anti-TNF- α monoclonal antibodies to promote the development of regulatory M2 macrophages, which are induced by infliximab in responder, but not in non-responder, IBD patients (Vos *et al.* 2012).

Proteolytic digestion by MMP-3 and MMP-12 leads to the complete removal of the Fc region of anti-TNF- α agents. Following exposure to MMP-3 or MMP-12, infliximab and adalimumab are still able to neutralise soluble TNF- α as F(ab')₂ fragments, even at the lowest anti-TNF- α /MMP concentration *ratio* of 1:10. However, proteolytic degradation of anti-TNF- α agents may be clinically relevant in IBD patients due to the consequent impairment of Fc-mediated properties. Moreover, since binding of the IgG to the FcRn has been shown to protect them from catabolism (Akilesh *et al.* 2007), the removal of the Fc region from anti-TNF- α agents operated by MMP-3 and MMP-12 may result in decreased serum trough levels of these drugs. Upon exposure to IBD mucosal protein extracts, infliximab and adalimumab, at low concentration, display an impaired ability to neutralise soluble TNF- α .

Human IgG₂ are resistant to proteolytic cleavage by MMPs, however their Fc-dependent immunologic functions are weak (Brezski *et al.* 2011). Specific

mutations can be incorporated into the Fc region of protease-resistant monoclonal IgG₁ antibodies, resulting in enhanced cell killing properties (Kinder *et al.* 2013). Therefore, it may be possible to apply the same strategy in order to generate anti-TNF- α agents resistant to proteolytic cleavage, without compromising their immunologic properties.

Our findings provide an explanation for the failure in CD of etanercept (Sandborn *et al.* 2001), which is highly susceptible to proteolytic cleavage. Etanercept is the only anti-TNF- α agent, among those studied, that loses its TNF- α neutralisation ability after Fc removal by MMP-3 and MMP-12, and that, even at high concentrations, loses its TNF- α neutralisation ability after exposure to IBD mucosal protein extracts. The effect of MMP-3 and MMP-12 on the ability of etanercept to neutralise TNF- α may be related to a conformational change in the p75 TNF receptor when cleaved from the IgG Fc. Conversely, the effect of IBD mucosal protein extracts on the ability of etanercept to neutralise TNF- α may depend on the possible degradation of the p75 TNF receptor itself by different proteolytic enzymes present in IBD mucosa.

Nevertheless, etanercept is clinically useful in rheumatoid arthritis (Weinblatt *et al.* 1999), and there are many reports showing that MMP-3 is elevated in the rheumatoid joint (Vandooren *et al.* 2004). However, concentration and activity of MMPs and their natural inhibitors in the affected tissues may be different in different diseases affecting different tissues, therefore local quantification of proteolytic activity would be important for interpretation of our results and translation into practical consequences. Nevertheless, in diseases such as IBD, cleavage and loss of function of biologic agents may be quantitatively greater than in the rheumatoid joint. The high susceptibility of etanercept to proteolytic cleavage suggests that there may be an inherent problem with Fc receptor fusion proteins at sites of inflammation rich in proteases. The failure of the cytotoxic T lymphocyte antigen (CTLA)-4-IgG fusion protein abatacept both in CD and in UC (Sandborn *et al.* 2012a) supports this idea.

The addition of the specific MMP-3/MMP-12 inhibitor UK370106 to the cleavage reactions prevented both MMP-3- and MMP-12-induced loss of TNF- α neutralisation ability of etanercept. Conversely, the broad-spectrum MMP inhibitor marimastat was able to restore TNF- α neutralisation ability of etanercept following exposure to MMP-3, but not MMP-12, in keeping with its known activity profile. On the other hand, marimastat showed a stronger effect than UK370106 in restoring TNF- α neutralisation ability of anti-TNF- α agents exposed to IBD mucosal protein extracts, supporting the view that multiple proteases contribute to the cleavage process in the inflamed mucosa.

Biomarker studies on IgG cleavage in IBD sera

In our study, we were particularly interested in exploring proteolytic degradation as a possible mechanism underlying lack of response to anti-TNF- α therapy in IBD. However, the demonstration that this mechanism is biologically plausible was the starting point for investigating the possibility to develop a biomarker, which may ultimately help identify the subset of IBD patients with the highest likelihood of responding to anti-TNF- α agents.

Different IBD patients are characterised by extensive disease heterogeneity, which involves genetic, immunologic, and clinical aspects. There are considerable ongoing efforts in order to develop biomarkers involving different clinical aspects of IBD. As an example, gene array profiling has been used to identify IBD patients with poor prognosis. In particular, based on transcriptional signatures of circulating CD8⁺ T cells, it is possible to identify at diagnosis, both in CD and in UC, the subgroup of patients with higher incidence of relapses (Lee *et al.* 2011). Regarding anti-TNF- α therapy in IBD, biomarkers capable of predicting patients who will or will not respond to treatment are not currently available in the clinical practice. Patients with high serum concentrations of CRP show significantly higher responsiveness and remission rates compared to those

with normal CRP (Jürgens *et al.* 2011). A decrease in faecal calprotectin early after the start of infliximab treatment predicts remission in UC patients (De Vos *et al.* 2012), however this can only be evaluated after the start of anti-TNF- α treatment. One gene array profiling study in UC has identified a mucosal gene expression panel able to distinguish responders from non-responders to infliximab (Arijs *et al.* 2009), and another gene expression profiling study based on TNF- α signalling has allowed discrimination between responders and non-responders, both in UC and in CD (Westra *et al.* 2013).

In order to evaluate whether signs of anti-TNF- α degradation in IBD could be detected in the serum of patients, we tried to measure clipped infliximab in IBD patients' sera collected immediately after the first or before the second infusion, however these experiments were uniformly negative. This may be due to the low *ratio* of clipped *versus* intact drug within the sera collected one hour after the infusion, or to the catabolism of degraded IgG in the samples collected before the following administration. An additional reason may be the fact that the F(ab')₂ fragments generated as a result of proteolytic cleavage become self-antigens that are recognised by autoantibodies (Brezski *et al.* 2008). This may prevent the possibility to detect clipped infliximab with an immunoassay directed against the same epitope. Interestingly, patients with rheumatoid arthritis have a higher incidence of serum anti-hinge autoantibodies compared to control subjects (Rispen *et al.* 2012). We performed an ELISA on IBD and control sera upon coating the plate with the peptide sequence on the IgG hinge region generated by MMP-3/MMP-12 cleavage. We observed that sera from active IBD patients contain significantly higher concentrations of anti-hinge autoantibodies compared to control subjects, and that, overall, IBD patients who subsequently did not respond to treatment with anti-TNF- α agents have significantly higher levels of anti-hinge autoantibodies than responders.

Finally, we also hypothesised that, in parallel to exogenous IgG, as it is the case with anti-TNF- α agents, cleavage of higher quantities of endogenous IgG occurs

in the inflamed IBD intestinal mucosa compared to the normal mucosa of control subjects, and that this is reflected in the serum. We therefore performed an ELISA on IBD and control sera after absorbing on the plate the biotinylated antibody specific for the MMP-3 and MMP-12 cleavage sites on the hinge region of human IgG, and we observed that sera from active IBD patients contain significantly higher serum levels of MMP-3-/MMP-12-cleaved endogenous IgG than control subjects. We then stratified IBD patients according to their response to biologic therapy, and we observed that, overall, those who subsequently did not respond to treatment have significantly higher levels of MMP-3-/MMP-12-cleaved endogenous IgG than responders.

Limitations of the study and possible confounding factors

In our *in vitro* experiments we have only studied LPMC-derived MMP-3, MMP-12 and MMP-9, and we have not assessed the effects of a range of other proteases with known ability to cleave IgG, including IEC-derived MMP-7, MMP-13 or the serine proteases neutrophil elastase and cathepsin G (Brezski *et al.* 2009; Brezski *et al.* 2010), which may play a relevant role in biologic agent clipping *in vivo*. Moreover, in our experiments we used single doses of MMPs added at a single time and we do not know if these reflect the *in vivo* situation, where it is likely that there is continuous production of MMPs by myofibroblasts and inflammatory cells into the pericellular spaces.

The highest concentration of rhMMP-3 and rhMMP-12 (1 µg/ml) that we used in our *in vitro* experiments when we tested them as positive controls on the MMP-3/MMP-12 activity assay had a higher activity than IBD protein extracts. Nevertheless, in the MMP-3 and MMP-12 cleavage reactions for the integrity analysis and the protein G assay we used a range of rhMMP concentrations (0.001-1 µg/ml), which most likely include physiologically relevant activity levels in IBD inflamed mucosa. Moreover, in some of the Western blots of anti-TNF-α agents after incubation with MMP-3 and MMP-12, we observed a disparity

between the intensity of intact anti-TNF- α agents and the cumulative intensity of the fragments produced after MMP-3 and MMP-12 cleavage. We are unable to exclude that this could be due to additional cleavage at different sites than the known cleavage sequence in the hinge region of IgG₁, with consequent formation of fragments which may not be recognised by the anti-Fc antibody used as a primary antibody for immunoblotting.

In the protein G assay, only intact anti-TNF- α agents can bind to the protein G-coated plate, are then recognised by secondary antibodies against F(ab')₂ or p75 TNFR (chicken antibodies, which are unable to bind to protein G) and give a positive signal, therefore the optical density can be considered as an indication of the amount of intact anti-TNF- α agent. The reduction of positive signal observed upon MMP cleavage of anti-TNF- α agents suggests that the Fc region has been removed by MMP-3 and MMP-12. Based on our experiments, we cannot exclude that free/cleaved Fc fragments may interfere/compete with intact anti-TNF- α agents for binding to the plates, and a possible way to evaluate this would be to perform the protein G assay with Fc fragments (commercially available) plus intact anti-TNF- α agents at different concentrations. Nevertheless, if present, interference/competition by free/cleaved Fc fragments in the cleavage reaction product would amplify, rather than diminish, the possibility to detect and quantify Fc removal by MMPs in the protein G assay.

There is considerable heterogeneity in the MMP mucosal expression profile in different IBD patients, which requires further investigation. Moreover, the multiple bands detected after incubation of adalimumab and infliximab with IBD mucosal protein extracts do not correspond to the 32 kDa band seen with recombinant MMP-3 and MMP-12, suggesting that, in the complex situation of inflammation, clipping of biologic agents may be variable in different patients. The interpretation of the clinical relevance of our data regarding the effect of inflamed IBD mucosal protein extracts on anti-TNF- α agents may be confounded by the fact that we do not exactly know what proportion of systemically administered anti-TNF- α agents

access the intestinal mucosa. However, an indirect suggestion that anti-TNF- α agents exert their action in the intestinal mucosa is provided by the study by Atreya *et al.* (Atreya *et al.* 2014), who showed by confocal laser endomicroscopy that CD patients with high numbers of transmembrane TNF- α^+ cells in the colonic mucosa have a substantially better response rate to adalimumab compared to patients with low numbers of transmembrane TNF- α^+ cells in the colonic mucosa. Moreover, we do know that IBD mucosal protein extracts have a significantly higher MMP-3/MMP-12 activity compared to control protein extracts, without any difference between CD and UC patients, and that several other proteases are up-regulated in IBD inflamed mucosa. Further studies on the effects of proteolysis on the function of anti-TNF- α agents in IBD mucosa seem therefore appropriate.

Our data on serum levels of cleaved IgG and anti-hinge autoantibodies need to be interpreted with caution, as there is a high degree of overlap between the groups considered. At the same time, there is also a high degree of heterogeneity in the serum levels of both MMP-3-/MMP-12-cleaved endogenous IgG and anti-hinge autoantibodies within the same patient group, especially among IBD patients. Moreover, the sample size in our analysis, particularly with regards to the comparison between responder and non-responder IBD patients, is considerably small. Nevertheless, even among a small number of non-responder IBD patients, there is a subgroup with high levels of MMP-3-/MMP-12-cleaved endogenous IgG, which may be the biomarker of high mucosal proteolytic activity contributing to non-responsiveness to treatment. Moreover, it should be pointed out that there are multiple factors which can contribute to primary non-responsiveness to biologic therapy, and that ours was a pilot study on a small cohort of patients.

Future experiments

While our study has provided evidence that proteolytic degradation is a biologically plausible mechanism which may contribute to primary non-

responsiveness to anti-TNF- α agents in IBD by affecting their bioavailability in the intestinal mucosa, it forms the starting point for a number of future investigations.

In the present study we have not examined the possibility that proteolytic cleavage by inflamed IBD protein extracts may influence the efficacy of anti-TNF- α agents by means of *in vivo* experiments. This could instead be the focus of a future preclinical interventional study on the treatment of experimental colitis with anti-TNF- α antibodies combined with the broad-spectrum MMP inhibitor marimastat. Although this approach would not provide clear evidence that MMP-3 and MMP-12 are the key enzymes involved in proteolytic degradation, it will be useful in order to evaluate whether MMP inhibition may enhance the efficacy of anti-TNF- α therapy.

Furthermore, we will clarify the effects of other proteases with known ability to cleave IgG₁, including MMP-7, MMP-13, the serine proteases neutrophil elastase and cathepsin G, which may play a relevant role in biologic agent degradation *in vivo*, on the integrity and function of anti-TNF- α agents. Furthermore, we will investigate the effects of proteases on biologic agents with different mechanism of action that are becoming increasingly relevant in the treatment of IBD, such as the anti- $\alpha_4\beta_7$ integrin monoclonal antibody vedolizumab (Feagan *et al.* 2013; Sandborn *et al.* 2013b), the anti- α_7 integrin monoclonal antibody etrolizumab (Vermeire *et al.* 2014), and the anti-p40 monoclonal antibody ustekinumab (Sandborn *et al.* 2012b). We are also going to clarify the presence of a class-specific increased susceptibility of Ig Fc fusion proteins, such as etanercept and the CTLA-Ig abatacept, to proteolytic degradation.

In addition to soluble TNF- α neutralisation, anti-TNF- α agents are supposed to exert their therapeutic effect by inducing immune cell apoptosis by reverse signalling upon binding transmembrane TNF- α (Tracey *et al.* 2008). Although this is not an Fc-dependent property, it would be important to evaluate the effect of

MMP-3, MMP-12, and of other proteases with the potential ability to degrade also the F(ab'), on the ability of anti-TNF- α agents to induce apoptosis of immune cells. Moreover, in addition to the ability to promote the development of regulatory M2 macrophages (Vos *et al.* 2011), anti-TNF- α agents have been shown, at least *in vitro*, to exert important effects on immune cells by two other Fc-dependent properties, namely antibody-dependent cell-mediated cytotoxicity upon binding the IgG Fc receptor, and complement-dependent cytotoxicity (Nesbitt *et al.* 2007). We will investigate the effects of proteolytic cleavage on these properties of anti-TNF- α agents, which we have not evaluated in the present study, in a future confirmation study.

In our study we focused on serum markers of IgG proteolytic cleavage, and we did not perform *in situ* analysis of cleaved anti-TNF- α agents – or cleaved endogenous IgG – on colonic biopsy sections from responder and non-responder IBD patients. This, which can be performed by immunofluorescence using the same biotinylated antibody specific for the MMP-3 and MMP-12 cleavage sites on the hinge region of human IgG that we used to measure MMP-3-/MMP-12-cleaved endogenous IgG by ELISA, should be assessed in a future validation study. Moreover, since it is likely that, in addition to MMP-3 and MMP-12, other proteases with different cleavage site specificity contribute to anti-TNF- α and IgG cleavage in the inflamed intestinal mucosa, it would be important to characterise further and to quantify anti-TNF- α and IgG fragments in IBD inflamed mucosa and serum. It has been shown that faecal loss of infliximab through the ulcerated intestinal mucosa is associated with primary non-responsiveness in UC (Brandse *et al.* 2015), and it will be interesting to investigate the proportion of intact and cleaved anti-TNF- α in the stool of IBD patients, and to assess whether it correlates with response to biologic therapy.

Finally, therapeutic drug monitoring by serial measurement of serum trough levels of anti-TNF- α agents and anti-drug antibodies is a valuable strategy for optimising the management of non-responsiveness to biologic therapy in IBD

(Afif *et al.* 2010; Ben-Horin *et al.* 2014; Vande Casteele *et al.* 2015). However, there is a strong need to develop biomarkers able to stratify IBD patients according to their subsequent possibility to respond to biologic therapy. For this purpose, a large, prospective validation study is needed to explore the possibility to use the quantification of serum levels of cleaved IgG and anti-hinge autoantibodies as biomarkers of responsiveness to biologic therapy in IBD.

Chapter 6: Inflammatory skin and bowel disease linked to a deletion in *ADAM17*

Note: Genetic analysis, ADAM17 and ADAM10 expression studies in patients' skin and small intestine, and DSG expression and immune cell quantification in patient's skin (Paragraphs 6.4.1-3) have been performed by Professor David P Kelsell's group in the Centre for Cell Biology and Cutaneous Research laboratory, Blizard Institute, Barts and the London School of Medicine and Dentistry, London, UK. Primary data of these experiments are not available, therefore I could not show scale bars and magnification values on all micrographs, and I could not include immunohistochemical characterisation of B cells, natural killer cells, neutrophils and dendritic cells in the skin of the affected boy, as well as isotype control antibody stainings, which were however performed for all experiments. These results have been published in the following paper, on which I am second Author (Blaydon DC, Biancheri P, Di WL, Plagnol V, Cabral RM, Brooke MA, van Heel DA, Ruschendorf F, Toynbee M, Walne A, O'Toole EA, Martin JE, Lindley K, Vulliamy T, Abrams DJ, MacDonald TT, Harper JI, Kelsell DP. Inflammatory skin and bowel disease linked to ADAM17 deletion. *N Engl J Med* 2011;365:1502-8).

We performed genetic studies in a family of consanguineous parents and their three children, two of whom had the same clinical features, with skin and small intestine inflammatory lesions, and we identified a deletion in *ADAM17*. Further information on the background to this Chapter is reported in Paragraph "1.2 Proteases and their inhibitors" and in Section "1.4.6 Proteases in IBD intestinal mucosa".

6.1 Aims of the study

This study aims to explore the consequences of a deletion in *ADAM17*, identified in a family of consanguineous parents and their three children, on skin barrier function and pro-inflammatory immune response. We have used the following

approach:

5. Evaluation of ADAM17 expression in the skin and keratinocytes from the affected boy and control subjects, and in duodenal biopsy sections from the affected siblings and control subjects.
6. Assessment of pro-inflammatory cytokine production by PBMCs from the affected boy, the unaffected mother and control subjects.

6.2 Case report

Two out of three children born to consanguineous parents (first cousins) of Lebanese origin had the same clinical features involving the skin, hair, and small intestine. Both skin lesions and diarrhoea developed from the second day of life. Professor David P Kelsell and his research group are interested in cutaneous syndromes, and particularly those with a phenotypic indication of a desmosomal disorder, such as the hair phenotype. Moreover, they are also interested in helping to deliver a genetic diagnosis in difficult and rare cases. The skin, hair, and small bowel disorder affecting the boy could not be defined with a specific diagnosis, neither did the similar disorder of the affected girl. Thus, when approached by the Great Ormond Street Hospital, Professor David P Kelsell and his research group took on the challenge. The unknown diagnosis, together with the similarity between the disorder affecting the two siblings, and the consanguinity of the parents prompted to hypothesise that a genetic mutation could contribute to the syndrome.

The affected girl had died at 12 years of age from fulminant *Parvovirus B19*-associated myocarditis, and, on subsequent investigation, the affected boy was found to have left ventricular dilatation (end diastolic dimension 60 mm) with borderline systolic function (ejection fraction 55%).

The skin lesions were perioral and perianal erythemas with fissuring and a generalised pustular rash that developed into psoriasiform erythroderma, with flares of erythema, scaling, and widespread pustules (Fig. 6.1A, B). The skin of the affected siblings, in the first years of life, was prone to infection with *S. aureus*, resulting in recurrent blepharitis and *otitis externa*. Their hair was short or broken, and their eyelashes and eyebrows were wiry and disorganised (Fig. 6.1A). They had thickened nails, with frequent paronychia caused by *Candida* and *Pseudomonas* infections (Fig. 6.1C). Microscopic examination of the affected siblings' hair showed an unusual hair shaft abnormality with severe weathering

and a markedly damaged cuticle. A skin biopsy showed patchy vacuolation of the basal layer over much of the biopsy and pigment incontinence, with a perivascular lymphocytic infiltrate involving vessels of the papillary dermis.



Figure 6.1

Figure 6.1. Clinical features of the syndrome. The affected boy is shown at five years of age, with (A) facial erythema and scaling, short scalp hair, and disorganised, wiry eyebrows; (B) widespread pustules and erythema; and (C) swelling of the distal phalanges, abnormal nails, and a susceptibility to paronychia.

The chronic diarrhoea in the affected girl was associated, at four months of age, with failure to thrive. The diarrhoea was predominantly bloody with malabsorptive characteristics, worsening in parallel with increases in the severity of the skin disease and exacerbated by intercurrent gastrointestinal infections. The affected girl underwent gastroscopy at the age of three years and the duodenal biopsies revealed a plasma cell duodenitis and chronic gastritis. The affected boy underwent gastroscopy and colonoscopy at the age of six years, and duodenal

biopsies revealed a plasma cell duodenitis with crypt hyperplasia, villous atrophy and mucosal eosinophilia.

Both affected siblings had extensive immunological investigations, including IgG, IgA and IgM, Ig subclasses, vaccine responses (*Haemophilus influenzae B*, tetanus and pneumococcus), which were all normal with no evidence of an immunodeficiency. Both affected siblings did have moderately raised IgE levels.

Treatments included acitretin, ciclosporin, methotrexate and adalimumab, none of which produced a significant sustained improvement in the skin or bowel disease. The skin infections initially responded best to systemic antibiotics, but this has become problematic because of increased resistance and a limited choice of antibiotics.

6.3 Chapter-specific Materials and Methods

6.3.1 Patients and tissues

Skin samples were obtained from family members as well as control subjects undergoing cosmetic (facelift or “tummy tuck”) surgery, and blood specimens for collection of PBMCs were obtained from family members and control subjects. This study was approved by the NRES Committee London - City & East (REC reference: 08/H1102/73), and informed consent was obtained from the family members and control subjects. None of the control subjects were age- or sex-matched. It was not possible to study matched control subjects due to the age of the affected boy.

6.3.2 Study design

All family members (both parents, the affected boy, the affected girl and the unaffected boy) were genotyped and analysed by SNP-homozygosity mapping and targeted next-generation sequencing of the regions of linkage on the genome. Upon the identification of a deletion in *ADAM17*, the protein expression levels of ADAM17 were studied in the skin and keratinocytes from the affected boy and control subjects, and in duodenal biopsy sections from the affected siblings and control subjects. Moreover, the expression of desmogleins was investigated in the skin and keratinocytes from the affected boy and control subjects, and the distribution of immune cells was studied in the skin of the affected boy. Finally, peripheral venous blood was collected once from the affected boy and three male control subjects of the same age as the affected boy, and another time from the affected boy, the unaffected mother and three control subjects. PBMCs were isolated from peripheral blood samples and cultured with various stimuli, and subsequently pro-inflammatory cytokine levels were measured in culture supernatants. Unfortunately, the affected girl died before the start of this study, therefore it was only possible to study the expression of

ADAM17 in her duodenum on paraffin-embedded tissue sections of duodenal biopsies.

6.3.3 SNP mapping, targeted sequence capture and sequencing

Due to the consanguinity within the family, homozygosity mapping using Affymetrix 10K SNP arrays (Affymetrix, Santa Clara, CA, USA) was performed. All five family members (both parents, the affected boy, the affected girl and the unaffected boy) were genotyped and analysed using Allegro software (Gudbjartsson *et al.* 2005). Quality control and data conversion of the SNP array data was made with the software ALOHOMORA (Rüschendorf *et al.* 2005). All exons within regions of putative linkage (identified via UCSC and Ensembl) were then included on a sequence capture array (Roche Nimblegen Inc., Madison, WI). The DNA from the affected male was pooled with the DNA extracted from four irrelevant other individuals (from unrelated projects) and hybridised to the array. The subsequent DNA “library” was then sequenced by next generation sequencing on a GAII sequencer (Illumina Inc., San Diego, CA). Raw 76bp paired end fastq reads were aligned to the human reference sequence (hg19) using novoalign (www.novocraft.com), including the soft clipping, adapter trimming, and quality calibration options. Filtering for clonal reads, pileup generation and SNP calling were performed using in-house perl/C++ scripts. SNP calling used a Bayesian approach adapted to deal with the presence of multiple haplotypes in the pool. Insertion/deletion (indels) calls were based directly on the novoalign output and were filtered. Indels likely to be true events were called if found in at least three independent reads.

6.3.4 Immunofluorescence

Primary keratinocytes were isolated from skin biopsies for Western blotting and immunofluorescence staining was performed on frozen or paraffin-embedded tissue sections collected from skin and duodenal biopsies from the affected

siblings and control subjects. Immunofluorescence and Western blotting studies were performed using antibodies against ADAM17 (skin immunofluorescence: ab39161, Abcam Ltd; small intestine immunofluorescence: ab39163, Abcam Ltd; Western blotting: ab2051, Abcam Ltd), ADAM10 (AB19026, Millipore, Bedford, MA) and DSG1/2 (DG3.10, Zymed, Cambridge, UK). For ADAM17 staining, paraffin sections were de-waxed, antigen retrieved and blocked with 3% serum in PBS and avidin/biotin blocking solution (Vector Laboratories, Burlingame, CA). The sections were then incubated with rabbit anti-ADAM17 antibody in 1:150 dilutions for 16 hours at 4°C. Sections were further incubated with biotinylated anti-rabbit IgG antibody for 1 hour and FITC-streptavidin conjugates for 30 minutes. For DSG staining, frozen sections were fixed in 1:1 methanol:acetone incubated with the relevant antibody in 1:250 dilution for 16 hours at 4°C followed by incubation of anti-mouse or anti-rabbit IgG conjugated with FITC. Stained tissues were imaged using a Zeiss laser confocal microscopy 710 (Carl Zeiss, Welwyn Garden City, UK) and images were processed using Adobe Photoshop CS (Adobe Systems Inc, San Jose, CA).

6.3.5 PBMC culture

PBMCs were isolated and cultured at 37°C for 24 hours in 96-well plates (BD Biosciences) (2×10^5 cells/well), in the presence or absence of 0.1-1000 ng/ml LPS (InvivoGen, San Diego, CA), or 100 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) plus 500 ng/ml ionomycin (Sigma-Aldrich), or in anti-CD3-coated 96-well plates (BD Biosciences) with 1 µg/ml anti-CD28 antibody (eBioscience). In particular, in one first experiment PBMCs from the affected boy and three control subjects were cultured with 10-1000 ng/ml LPS or with anti-CD3/anti-CD28 antibodies, and another time, in a separate experiment, PBMCs from the affected boy, the unaffected mother and three control subjects were cultured with 0.1-1000 ng/ml LPS or with anti-CD3/anti-CD28 antibodies or PMA plus ionomycin. Subsequently, TNF- α , IL-1 β , IL-6 and IFN- γ concentrations were measured by ELISA in cell culture supernatants.

6.4 Results

6.4.1 Genetic analyses

Analysis of the SNP array data revealed putative linkage (maximum lod score of 1.8) to three large stretches of SNP homozygosity, seen in DNA from both affected siblings, on chromosomes 2, 5, and 21 (Fig. 6.2A). After ruling out nine plausible candidate genes by using standard Sanger sequencing (data not shown), we included probes for all exons from these three regions of the genome on a capture array. A total of 1468 exons corresponding to 439 kb of DNA were captured in the affected boy and sequenced. After ruling out known SNPs (accessing dbSNP and 1000 Genome project via the Seattle SNP website url: <http://pga.gs.washington.edu/>), we identified 22 nonsynonymous single-nucleotide variants in coding regions. In parallel, we assessed the sequence data for insertion-deletion variations and discovered a new deletion of 4 bp in exon 5 of *ADAM17* (c.603-606delCAGA) on chromosome 2 that segregated with the disease in this family (Fig. 6.2B, Fig. 6.3). The unaffected boy lacked this deletion. We were unable to find rare variants, predicted to result in loss of function, within *ADAM17* in the dbSNP database or the 1000 Genomes database. Bioinformatic analysis predicted that the mutation would introduce a frameshift and a premature stop codon (p.Asp201GlufsX11) separated by 10 codons. Thus, the mutation predicts a severely truncated protein consisting of the signal peptide and pro-domain of ADAM17 and lacking the catalytic domain, disintegrin domain, transmembrane segment, and cytosolic tail (Fig. 6.4). Given the likely deleterious nature of the 4 bp deletion identified in *ADAM17* and the role of ADAM17 in processing TNF- α , this was likely to be the disease-associated mutation and the rest of the study focussed on ADAM17.

Figure 6.2. Genetic studies. (A) Due to the consanguinity within the family, homozygosity mapping using Affymetrix 10K SNP arrays was performed. All five family members (both parents, the affected boy, the affected girl and the unaffected boy) were genotyped and analysed using Allegro software Linkage analysis performed on SNP array data using Allegro revealed three regions of linkage on chromosomes 2, 5 and 21. (B) All exons within regions of putative linkage were then included on a sequence capture array. Sanger sequence traces showing a new homozygous deletion of 4 bp in exon 5 of *ADAM17* (c.603-606delCAGA) on chromosome 2 that segregated with the disease in this family. The parents are both heterozygous carriers and the unaffected boy is wild-type.

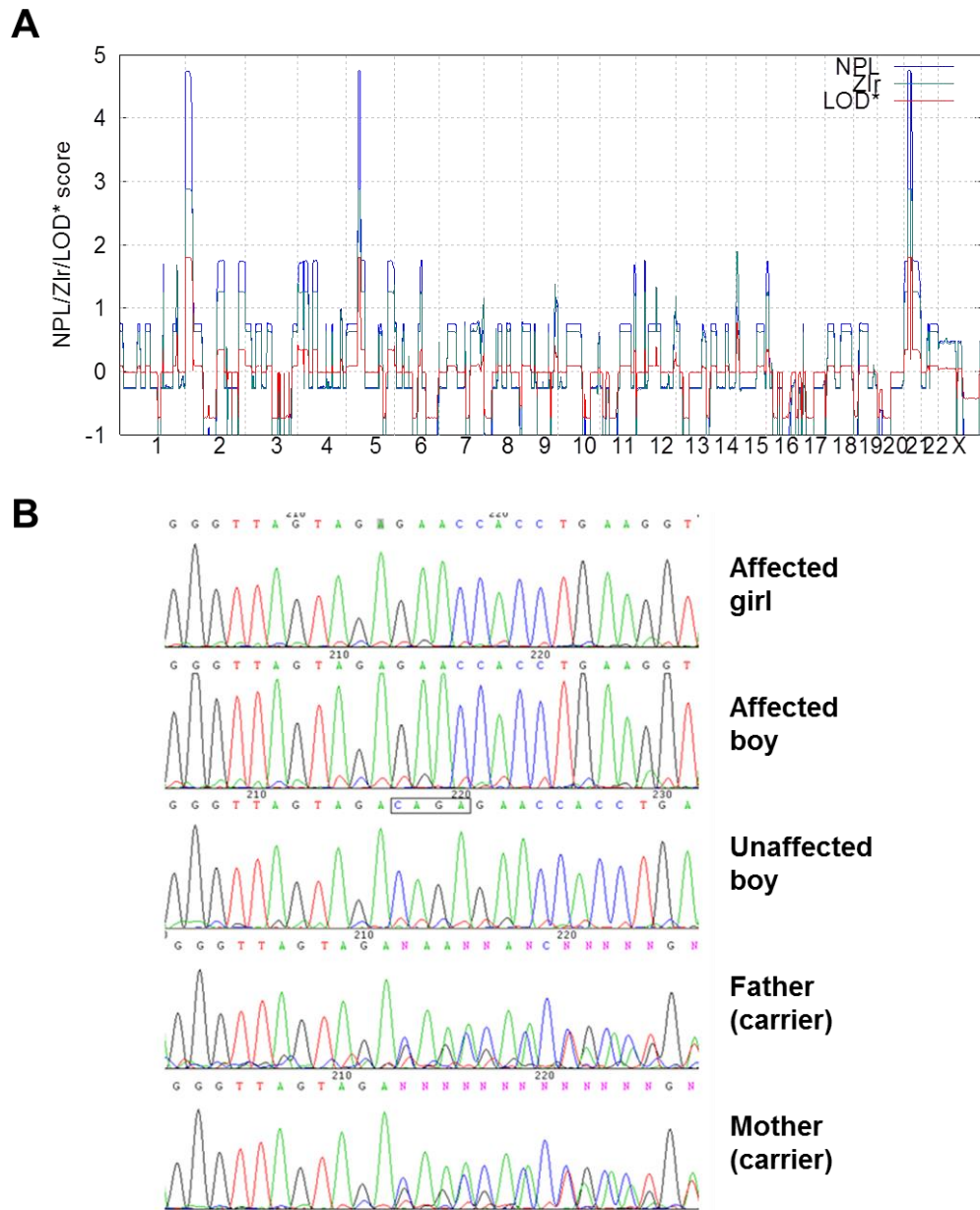


Figure 6.2

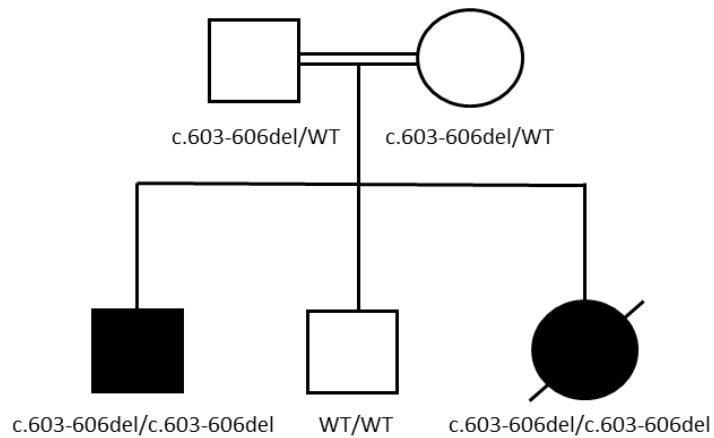


Figure 6.3

Figure 6.3. Family pedigree. The family pedigree shows the two affected siblings and the unaffected boy from a consanguineous marriage of first cousins; segregation of the *c.603-606delCAGA ADAM17* mutation is also shown. Squares and circles represent male and female family members, respectively; double horizontal lines indicate consanguinity; solid symbols and open symbols indicate affected and unaffected family members, respectively; and the slash indicates the deceased affected girl.

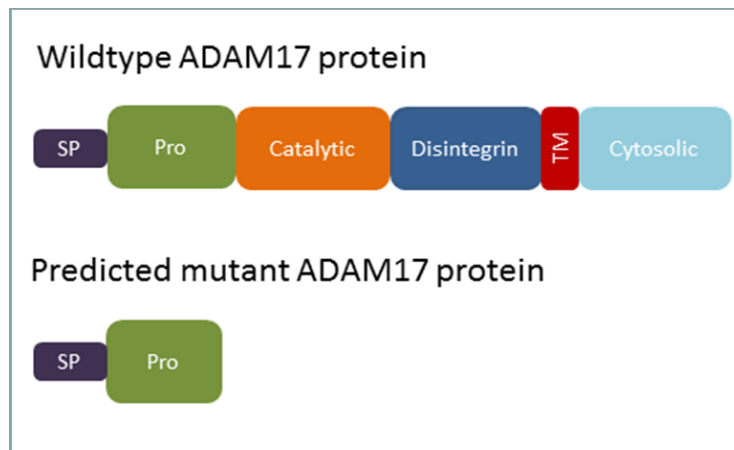


Figure 6.4

Figure 6.4. Predicted structure of wild-type and mutant ADAM17. Schematic illustrating the domain structure of wild-type ADAM17 protein (SP, signal peptide; Pro, pro-domain; catalytic domain; disintegrin domain; TM, transmembrane region; cytosolic tail) and the predicted structure of the p.Asp201GlufsX11 mutant protein if expressed.

6.4.2 ADAM17 expression studies in the affected boy's skin and in the affected siblings' duodenum

Immunofluorescence of control skin using an ADAM17 antibody which reacts with the pro-domain revealed expression throughout the epidermis with a cytoplasmic distribution (Fig. 6.5A). However, in the skin from the affected boy there was a marked reduction of ADAM17 expression (Fig. 6.5B).

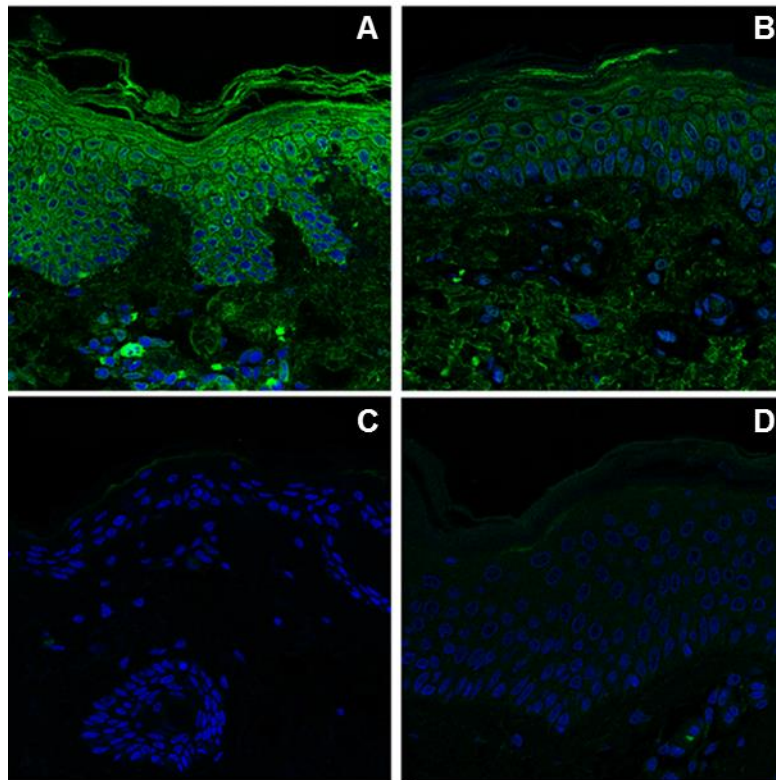


Figure 6.5

Figure 6.5. Expression of ADAM17 in the skin. Immunofluorescence staining with an ADAM17 antibody (green) that reacts with an epitope in the active site of ADAM17 (consisting of a cysteine switch and furin cleavage sites) was performed in paraffin-embedded sections of skin biopsies from the affected boy and one control subject. (A) In control skin ADAM17 is expressed throughout the epidermis, with a cytoplasmic staining pattern, whereas (B) ADAM17 expression is clearly reduced in the affected boy's skin. Control staining of the *nuclei* with 4',6-diamidino-2-phenylindole (DAPI; blue), without the addition of the ADAM17 antibody, is shown for (C) a section of control skin and (D) the affected boy's skin.

Histologic analysis and immunofluorescence for ADAM17 on duodenal biopsies from both affected siblings was performed. Compared with the normal findings in the control subject (Fig. 6.6A), in the affected girl there was evidence of a mononuclear cell infiltrate, villus blunting, and lengthening of crypts (Fig. 6.6B); although the findings were more variable, also in the duodenum of the affected boy there was evidence of a mononuclear cell infiltrate and villus blunting (Fig. 6.6C). ADAM17 was expressed in the enterocytes of the small intestine in a control subject (Fig. 6.6D), but was absent in the small intestine of the affected girl (Fig. 6.6E) and the affected boy (Fig. 6.6F).

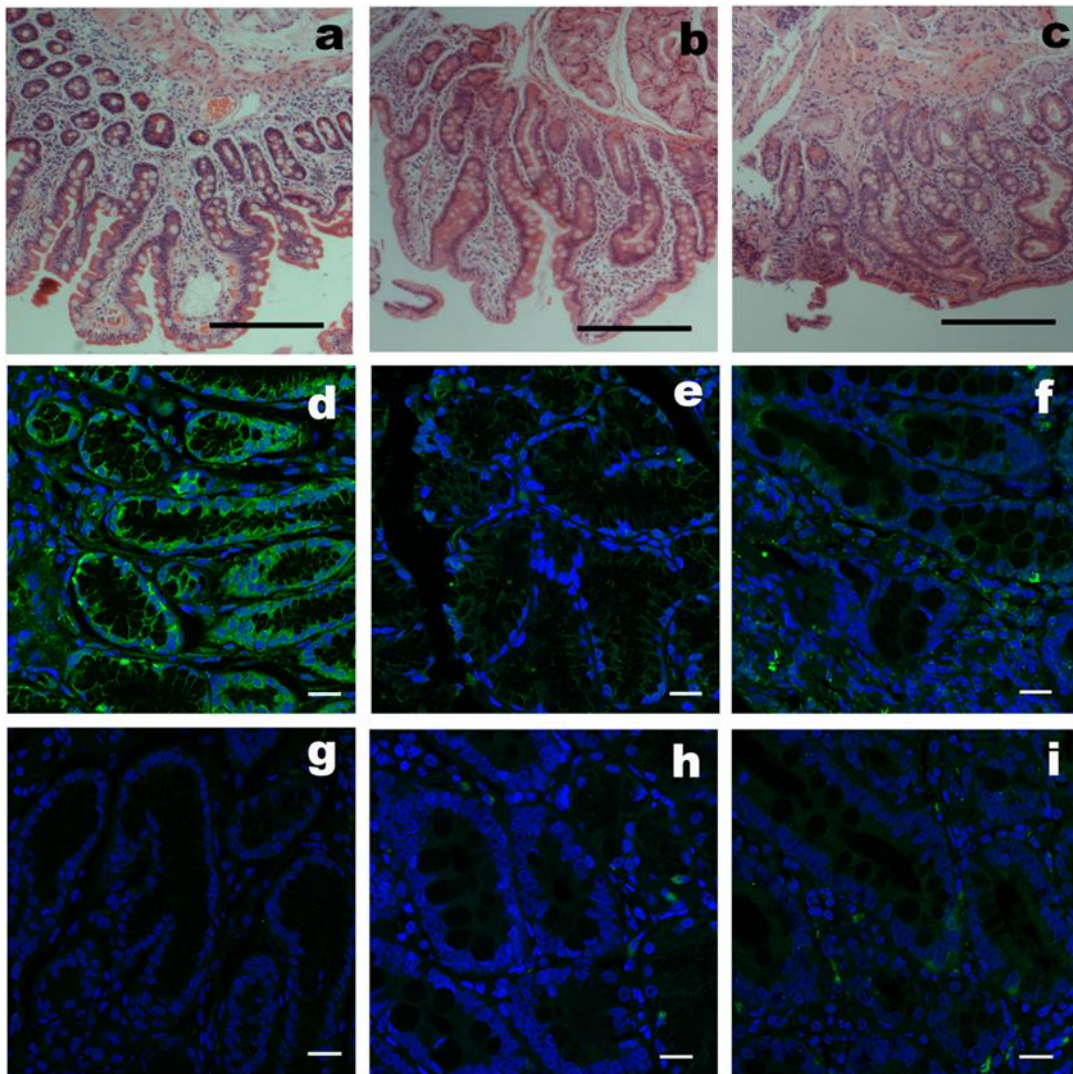


Figure 6.6

Figure 6.6 (see previous page). **Expression of ADAM17 in the duodenal mucosa.** (A-C) Haematoxylin and eosin staining of duodenal biopsies was performed in a control subject and in the two affected siblings. (D-I) Immunofluorescence staining with an ADAM17 antibody (green) that reacts with an epitope in the active site of ADAM17 (consisting of a cysteine switch and furin cleavage sites) was performed on duodenal biopsies from a control subject and the two affected siblings. (G-I) Control staining of the *nuclei* with 4',6-diamidino-2-phenylindole (DAPI; blue), without the addition of the ADAM17 antibody, is shown. (A-C) Scale bar = 250 μ m. (D-I) Scale bar = 20 μ m.

Furthermore, Western blotting of both PBMCs and keratinocyte lysates obtained from the affected boy showed an absence of ADAM17 expression, in contrast with the findings in control subjects and the unaffected mother (Fig. 6.7A). Of note, PBMCs from the unaffected mother showed lower ADAM17 expression, which may reflect her being heterozygous carrier of the ADAM17 mutation, however it is not possible to exclude that it is the result of interindividual variability. The expression of ADAM10, whose substrates overlap with those of ADAM17, was similar in keratinocytes from the affected boy and from a control patient undergoing cosmetic surgery (Fig. 6.7B).

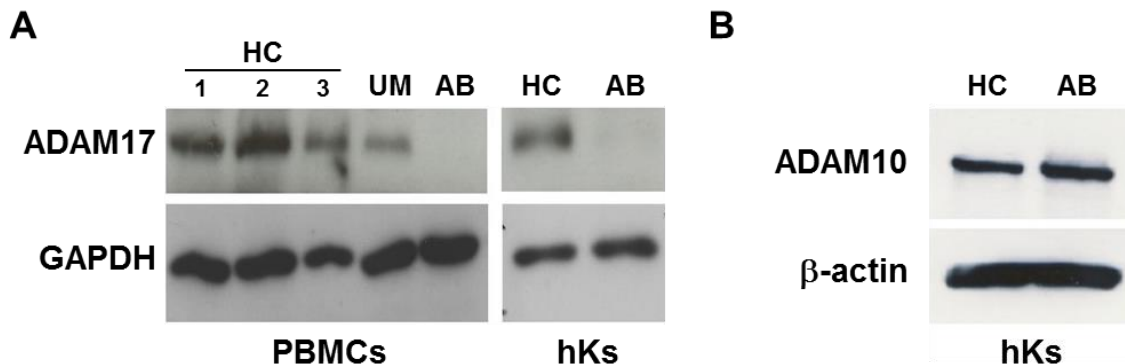


Figure 6.7

Figure 6.7. ADAM17 and ADAM10 expression in keratinocytes. (A) Western blotting for ADAM17, with the use of an antibody that reacts with the C-terminal of ADAM17, on lysates of PBMCs from three control subjects (HC), from the affected boy (AB) and the unaffected mother (UM), and on lysates of primary human keratinocytes (hKs) from one HC and the AB. Blots were stripped and analysed for GAPDH as an internal loading control. (B) Western blotting for ADAM10 on lysates of primary hKs from one HC and the AB. Blots were stripped and analysed for β -actin as an internal loading control.

6.4.3 Epidermal barrier and immune cell investigations on the affected boy's skin

ADAM17 functions as a sheddase with the ability to cleave and thereby release a multitude of different membrane-bound substrates, including desmogleins (DSGs), from the cell surface. Immunofluorescence analysis of skin sections and Western blotting of primary keratinocytes showed that expression of DSG (DSG1, DSG2, or both) is greater in the affected boy than in one control subject (Fig. 6.8), suggesting that DSG is retained on the plasma membrane in the control subject studied.

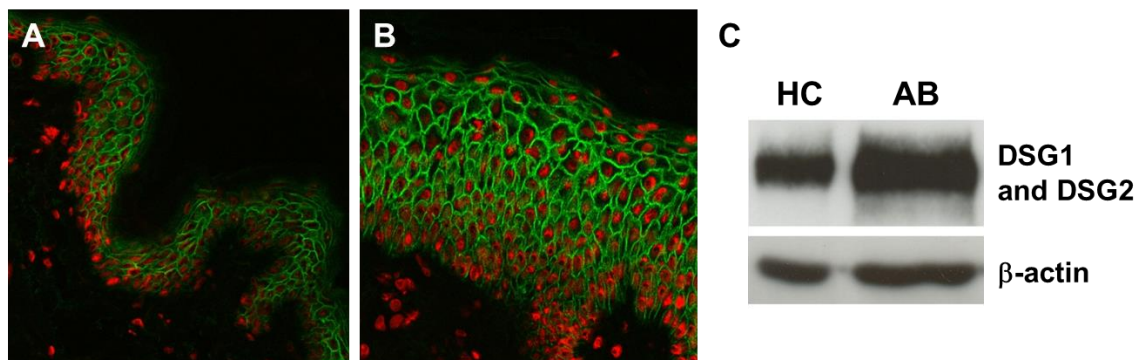


Figure 6.8

Figure 6.8. DSG expression in the skin. Immunofluorescence staining for DSG1 and DSG2 (green) was performed on frozen skin sections obtained from (A) a control subject and (B) the affected boy. Nuclei were stained with propidium iodide (red). (C) Western blotting for DSG1 and DSG2 on protein lysates of primary human keratinocytes from one control subject (HC) and the affected boy (AB). Blots were stripped and analysed for β -actin as an internal loading control.

Further immunohistochemical characterisation of the affected boy showed an infiltrate of T cells ($CD3^+$) around the skin follicles and in the epidermis. We observed $CD4^+$ T cells in the perifollicular region and $CD8^+$ T cells in the epidermis at the neck of the follicle (Fig. 6.9). There were some B cells ($CD20^+$), natural killer cells ($CD56^+$), or neutrophils ($elastase^+$) present, and levels of dendritic cells ($S100^+$) were normal (negative data not shown).

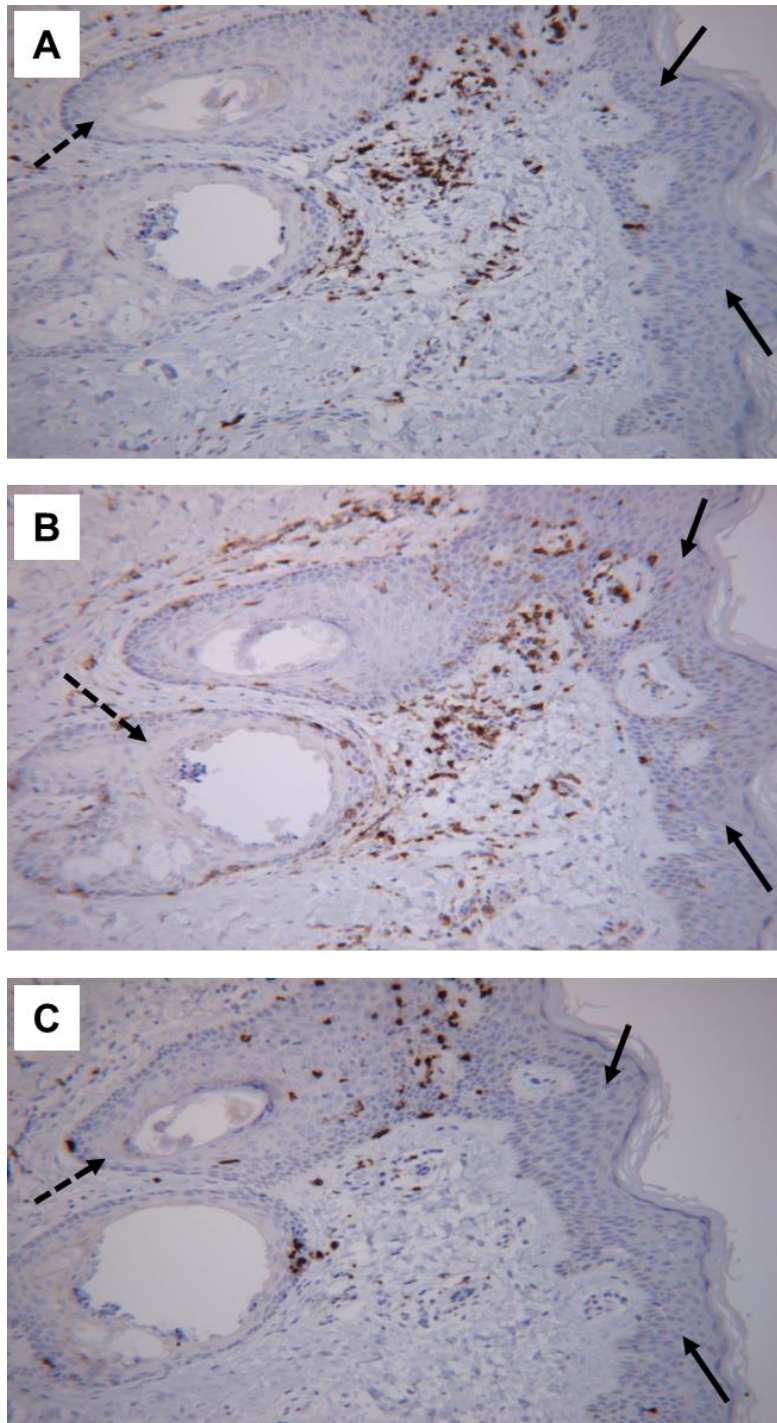


Figure 6.9

Figure 6.9. Expression of T cell markers in the skin of the affected boy. Immunohistochemistry revealed (A) anti-CD3-stained, (B) anti-CD4-stained and (C) anti-CD8-stained cells in the skin of the affected boy. Continuous arrows indicate the epidermis, dashed arrows indicate skin follicles.

6.4.4 Cytokine production by PBMCs

ADAM17 converts membrane-bound TNF- α into soluble TNF- α . Bearing in mind the inflammatory aspect of the disease phenotype, cytokine production by PBMCs isolated from the affected boy, his unaffected mother and three control subjects was investigated (Fig. 6.10). Due to limited availability of blood samples from the affected patient and the unaffected mother, only a total of two cell stimulations could be performed, with the second one being more extensive, with additional conditions of culture. Due to the low number of replicates ($n=1$ or $n=2$) of stimulation performed on PBMCs from the affected boy and the unaffected mother, statistical analysis could not be done. For the conditions with more than one experimental replicate, the mean cytokine concentration in pg/ml \pm SEM is reported below, whereas in case a condition was tested only once, the cytokine concentration in pg/ml was reported. TNF- α concentrations in the supernatants of PBMCs from the affected boy cultured with 0.1, 1, 10, 100 or 1000 ng/ml LPS were 168; 184; 399 ± 128 ; 386 ± 148 ; 405 ± 119 , respectively. TNF- α concentrations in the supernatants of control PBMCs cultured with 0.1, 1, 10, 100 or 1000 ng/ml LPS were 785 ± 59 ; 956 ± 72 ; 1128 ± 86 ; 1381 ± 85 ; 1465 ± 92 , respectively. TNF- α concentrations in the supernatants of PBMCs from the unaffected mother cultured with 0.1, 1, 10, 100 or 1000 ng/ml LPS were 580; 1256; 1452; 1693; 1843, respectively. TNF- α concentrations in the supernatants of PBMCs from the affected boy, the unaffected mother and control subjects cultured with medium alone were 0 pg/ml. IL-1 β concentrations in the supernatants of PBMCs from the affected boy cultured with 0.1, 1, 10, 100 or 1000 ng/ml LPS were 1418; 2503; 4620 ± 1433 ; 4788 ± 1610 ; 4875 ± 1298 , respectively. IL-1 β concentrations in the supernatants of control PBMCs cultured with 0.1, 1, 10, 100 or 1000 ng/ml LPS were 1213 ± 262 ; 1436 ± 334 ; 1599 ± 282 ; 2050 ± 365 ; 2214 ± 369 , respectively. IL-1 β concentrations in the supernatants of PBMCs from the unaffected mother cultured with 0.1, 1, 10, 100 or 1000 ng/ml LPS were 2733; 3138; 3264; 3749; 3870, respectively. IL-1 β

concentration in the supernatants of PBMCs from the affected boy cultured with medium alone was 141 ± 141 pg/ml. IL-1 β concentrations in the supernatants of PBMCs from the unaffected mother and control subjects cultured with medium alone were 0 pg/ml. IL-6 concentrations in the supernatants of PBMCs from the affected boy cultured with 0.1, 1, 10, 100 or 1000 ng/ml LPS were 5556; 6390; 9315 ± 1219 ; 11440 ± 864.0 ; 14270 ± 1650 , respectively. IL-6 concentrations in the supernatants of control PBMCs cultured with 0.1, 1, 10, 100 or 1000 ng/ml LPS were 2095 ± 447 ; 3159 ± 573 ; 2347 ± 792 ; 3505 ± 850 ; 4671 ± 1137 , respectively. IL-6 concentrations in the supernatants of PBMCs from the unaffected mother cultured with 0.1, 1, 10, 100 or 1000 ng/ml LPS were 5711; 7043; 9260; 9532; 10532, respectively. IL-6 concentrations in the supernatants of PBMCs from the affected boy cultured with medium alone were 2926 ± 2926 pg/ml. IL-6 concentrations in the supernatants of PBMCs from the unaffected mother and control subjects cultured with medium alone were 0 pg/ml. In summary, stimulation with LPS induced a strong and concentration-dependent increase in TNF- α production in PMBCs from controls and from the unaffected mother, whereas the increase in TNF- α production elicited by LPS in PBMCs from the affected boy was at least three-fold smaller. PBMCs from all samples showed similarly increased production of IL-1 β and IL-6 after LPS stimulation (Fig. 6.10A).

TNF- α concentrations in the supernatants of PBMCs from the affected boy, his unaffected mother and control subjects cultured with anti-CD3/CD28 antibodies were 1180 ± 343 , 10581 and 8809 ± 911 , respectively. IFN- γ concentrations in the supernatants of PBMCs from the affected boy, his unaffected mother and control subjects cultured with anti-CD3/CD28 antibodies were 9774 ± 654 , 12920 and 7458 ± 532 , respectively. TNF- α concentrations in the supernatants of PBMCs from the affected boy, his unaffected mother and control subjects cultured with PMA plus ionomycin were 639, 8695 and 5725 ± 1055 , respectively. IFN- γ concentrations in the supernatants of PBMCs from the affected boy, his

unaffected mother and control subjects cultured with PMA plus ionomycin were 5320, 9293 and 5149 ± 894 , respectively. In summary, stimulation with anti-CD3/CD28 antibodies evoked strong TNF- α production in PMBCs from controls and from the unaffected mother, whereas the increase in TNF- α production elicited by LPS in PMBCs from the affected boy was more than seven times smaller. PMBCs from all samples showed similarly high levels of IFN- γ production after stimulation with anti-CD3/CD28 antibodies (Fig. 6.10B). Shedding of transmembrane proteins including TNF- α on immune cells has been shown to be stimulated by activators of protein kinase C, such as PMA (Peschon *et al.* 1998). In response to PMA and ionomycin, similarly high amounts of IFN- γ were secreted by PMBCs from all samples, whereas TNF- α production by PMBCs was more than eight times smaller in the affected boy than in the control subjects and the unaffected mother (Fig. 6.10C).

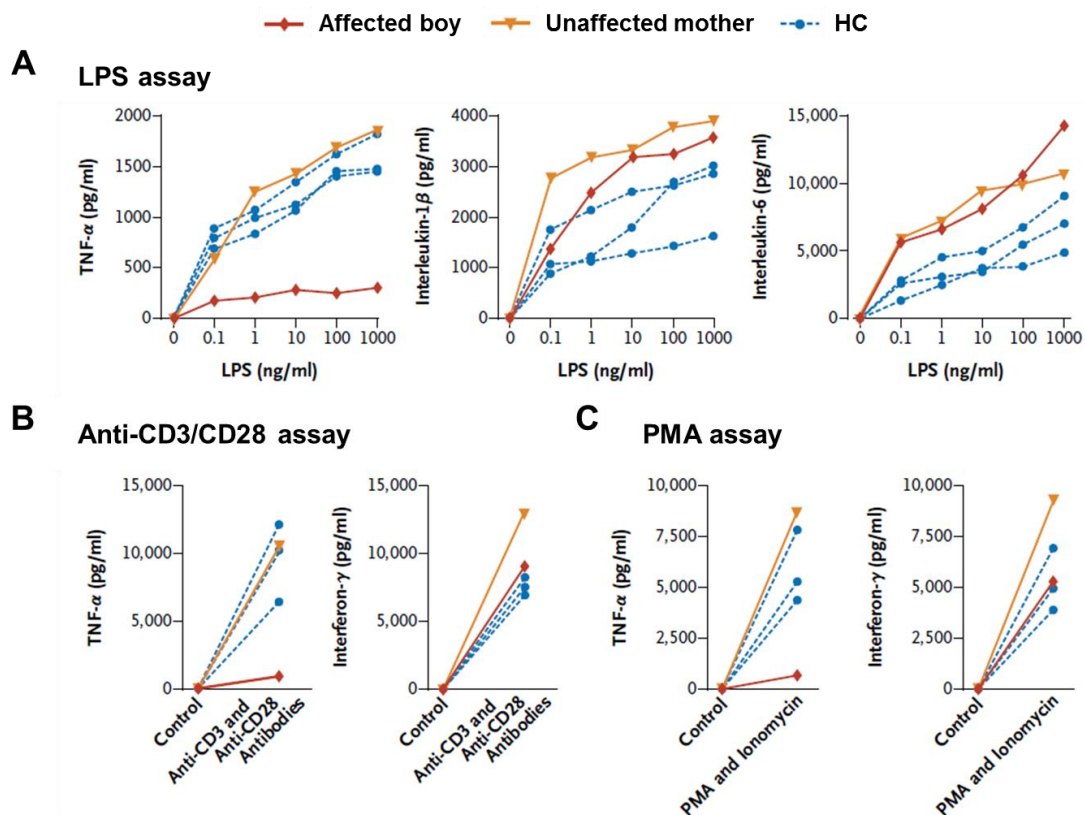


Figure 6.10

Figure 6.10 (see previous page). **Cytokine production by PBMCs.** Pro-inflammatory cytokine production by PBMCs from the affected boy, his unaffected mother, and three control subjects (HC) was evaluated. **(A)** Production of TNF- α , IL-1 β and IL-6 by PBMCs cultured for 24 hours in medium alone or LPS at concentrations of 0.1 to 1000 ng/ml. **(B)** Production of TNF- α and IFN- γ by PBMCs cultured for 24 hours with medium alone (Control) or in the presence of anti-CD3/CD28 antibodies. **(C)** Production of TNF- α and IFN- γ by PBMCs cultured for 24 hours with medium alone (Control) or in the presence of PMA plus ionomycin. Cytokine levels were measured in cell culture supernatants by ELISA. A total of two cell stimulations were performed due to limited availability of blood samples from the affected patient and the unaffected mother. All conditions of culture were tested in duplicate. Data displayed here, expressed as pg/ml, are those of the second experiment, and are representative of data relative to the same condition of culture (medium alone, 10-1000 ng/ml LPS and anti-CD3/anti-CD28 antibodies) performed in the first experiment on PBMCs from the affected boy and HC.

6.5 Results summary

In the current study, we found that:

12. Compared to the unaffected mother and control subjects, ADAM17 expression is down-regulated in the skin and keratinocytes from the affected boy, and in duodenal biopsy sections from the affected siblings.
13. After LPS stimulation, PBMCs from the affected boy produced comparable amounts of IL-1 β and IL-6, but substantially less soluble TNF- α compared to the unaffected mother and control subjects. After stimulation with anti-CD3/CD28 antibodies or PMA plus ionomycin, PBMCs from the affected boy produced comparable amounts of IFN- γ , but substantially less soluble TNF- α compared to the unaffected mother and control subjects.

6.6 Discussion

Our findings indicate that homozygous deletion in *ADAM17* gene in humans is associated with a complex, neonatal-onset, multi-organ syndrome affecting mainly the intestine, the skin, and the cardiovascular system. In this condition, *ADAM17* expression is down-regulated in the skin and in duodenal biopsy sections, and soluble TNF- α release by PBMCs is substantially reduced compared to control subjects. This Section focuses on the interpretation of our findings in the context of the existing literature, on the limitations of our study, and on possible future studies to be performed on newly identified humans with genetic *ADAM17* deficiency, in order to better understand the consequences of *ADAM17* dysregulation, both at the systemic and the mucosal levels.

ADAM17 homozygous mutations in humans and mice

Inflammatory disorders of the skin and the intestine, including eczema, psoriasis and IBD have been linked to changes in barrier function and immune responses, by means of genetic and functional studies. Large case-control studies combined with GWAS have identified common genetic risk factors, with low penetrance, for a plethora of human disorders. Such studies have also identified numerous SNPs in some of the genes linked to the regulation of immunity and inflammation affecting epithelial tissues (Liu *et al.* 2008; Dubois *et al.* 2010). High-throughput sequence technology can be used to identify rare but penetrant disease-associated mutations in affected members of families with Mendelian conditions (Ng *et al.* 2010; Rehman *et al.* 2010; Volpi *et al.* 2010; Kahrizi *et al.* 2011).

We combined high-throughput sequence technology with SNP-homozygosity mapping and targeted sequence capture to investigate likely causative genes in a syndrome of neonatal-onset inflammatory skin and bowel disease in two affected siblings born from consanguineous parents, and we identified the first described human mutation in the *ADAM17* gene. The mutation is predicted to

lead to a truncated protein that lacks all functional domains, including the catalytic domain required for the sheddase function of ADAM17. We suggest that the deletion mutation in *ADAM17* present in the homozygous state in both affected siblings is a major contributor to their disease. The existence of the syndrome has been confirmed by the study by Bandsma *et al.* (Bandsma *et al.* 2015), who reported the case of a child born from non consanguineous parents, who presented with severe diarrhoea, skin rash, and recurrent episodes of sepsis, eventually leading to her death at the age of 10 months. In this case, a different homozygous frameshift mutation in *ADAM17*, leading to a premature stop codon, has been identified. Also in this case the mutation has been predicted to lead to a truncated ADAM17 or to the absence of ADAM17 protein as a consequence of nonsense-mediated mRNA decay (Bandsma *et al.* 2015).

ADAM17 is a transmembrane metalloproteinase that exerts an important influence on development and disease via its effects on the EGF receptor (EGFR) and the TNF- α signalling pathways. In particular, ADAM17 converts transmembrane TNF- α into soluble TNF- α , but it can also cleave both TNFR1 and TNFR2 (Bell *et al.* 2007). Moreover, a substantial proportion of substrates on which ADAM17 exerts its sheddase activity are molecules with important immune functions (Scheller *et al.* 2011). ADAM17 cleaves adhesion molecules, including intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and L-selectin, thereby limiting the adhesion of immune cells to the endothelium, and it has been shown that leukocyte recruitment in the inflamed peritoneum is up-regulated in mice expressing non-cleavable L-selectin (Venturi *et al.* 2003). ADAM17 can cleave MHC class I-related chain A/B and B7-H6, which are ligands for receptors expressed on NK, NKT and cytotoxic T cells, thereby possibly contributing to the escape from immunosurveillance by cancer cells (Schlecker *et al.* 2014). Finally, IL-6 receptor (IL-6R) is another important substrate of ADAM17 sheddase activity (Boutet *et al.* 2009; Schumacher *et al.* 2015). In particular, IL-6R is only expressed on hepatocytes and leukocytes, whereas the transmembrane protein gp130 is ubiquitously expressed. Upon

cleavage, soluble IL-6R can bind IL-6, and this complex may subsequently bind gp130 and promote intracellular signalling in cell types that otherwise would not respond to IL-6 (Rose-John 2012). This process is known as trans-signalling, and it is particularly important for the pleiotropic functions of IL-6.

Mice with a homozygous mutation in the zinc-binding sequence of ADAM17/TACE (*tace*^{ΔZn/ΔZn} mice), which is essential for its metalloproteinase action, display a severely impaired survival rate. In particular, evaluation of the genetic composition of the mouse population between two-three weeks of age revealed that live mice with homozygous *tace*^{ΔZn/ΔZn} mutation are only 1.3% of total mice, as opposed to the expected Mendelian *ratio* of 25%, with the majority of homozygous *tace*^{ΔZn/ΔZn} mice dying between embryonic day 17.5 and the first day after birth (Peschon *et al.* 1998). All *tace*^{ΔZn/ΔZn} mutant fetuses at embryonic day 17.5 have open eyelids, resulting from a failure of eyelid fusion, and lack a conjunctival sac. The few *tace*^{ΔZn/ΔZn} mice which survive for several weeks show a 20-40% reduction in body weight compared to wild-type littermates, and display corneal inflammation, perturbed hair coats, and curly *vibrissae* (Peschon *et al.* 1998). Histologic sections of *tace*^{ΔZn/ΔZn} skin show disorganised distribution and structures of hair follicles, and histologic analysis of *tace*^{ΔZn/ΔZn} mutant fetuses at embryonic day 17.5 shows delayed or impaired maturation in multiple organs. In particular, the proximal small intestinal epithelium displays blunted *villi* and a hypercellular, pseudostratified mucosal epithelium, with altered cell polarity (Peschon *et al.* 1998). Lethality in *tace*^{ΔZn/ΔZn} mutant mice can not be attributed to soluble TNF-α deficiency, as mice lacking TNF-α show a normal development (Pasparakis *et al.* 1996). The *tace*^{ΔZn/ΔZn} mutation is also lethal in TNFR-deficient mice, indicating that the survival impairment is not due to inappropriate signalling by uncleaved transmembrane TNF-α. Indeed, this extensively abnormal phenotype has been attributed to the effects of TACE on a plethora of other molecules, including TGF-α and EGF (Peschon *et al.* 1998).

The hair, skin and eye defects observed in mice with *tace* ^{Δ Zn/ Δ Zn} mutation are similar to those reported in mice lacking TGF- α . In particular, ADAM17 cleaves transmembrane TGF- α to soluble TGF- α , which appears to be essential for hair follicle development and eyelid fusion *in vivo* (Luetteke *et al.* 1993; Mann *et al.* 1993). The epithelial maturation defects observed in mice with *tace* ^{Δ Zn/ Δ Zn} mutation are similar to those of mice lacking EGFR (Miettinen *et al.* 1995), which is the obligate receptor for several ligands cleaved by ADAM17, including EGF, TGF- α and amphiregulin (Scheller *et al.* 2011). In particular, *EGFR*^{-/-} mice suffer from impaired development in several organs, including the skin, the lungs, and the gastrointestinal tract (Miettinen *et al.* 1995).

Using exon-induced translational stop strategy, mice with a marked reduction, but not complete deficiency, of ADAM17 in all tissues (*ADAM17*^{ex/ex} mice) have been developed (Chalaris *et al.* 2010). *ADAM17*^{ex/ex} mice are viable, and have skin, hair and eye defects similar to those of TGF- α -deficient mice. Despite not showing overt histologic intestinal abnormalities, *ADAM17*^{ex/ex} mice are more susceptible to DSS-induced colitis than wild-type mice (Chalaris *et al.* 2010). Mice with an N-ethyl-N-nitrosourea-induced mutation in ADAM17 (wavedX mice) have also been developed, and express predominantly immature ADAM17, with impaired sheddase activity. Intestinal histology of wavedX mice is normal, however, similarly to *ADAM17*^{ex/ex} mice, they also have a higher susceptibility to DSS-induced colitis than wild-type mice (Brandl *et al.* 2010).

Association of human ADAM17 mutations with a multi-organ syndrome

When considering the affected siblings described in our study, it would appear that, differently from mice, in humans homozygous ADAM17 deficiency is compatible with survival. However, the affected girl in our study died at 12 years of age, and in the case reported by Bandsma *et al.* (Bandsma *et al.* 2015) the affected child, despite showing considerable similarities with the cases described in our study, died at the age of 10 months for respiratory insufficiency related to a

respiratory syncytial virus infection. This suggests that it is premature to draw any conclusion on the probability of long-term survival in this syndrome on the basis of the three cases described so far.

Both the affected siblings in our study had diarrhoea from the second day of life, which later spontaneously resolved, and also in the study by Bandsma *et al.* (Bandsma *et al.* 2015) the affected child developed watery diarrhoea during the first week of life, which did not disappear after discontinuation of feeding. It has been suggested that, similarly to DSS-induced colitis in *ADAM17^{ex/ex}* and wavedX mice, a trigger may have been necessary to induce diarrhoea in early childhood in the affected siblings described in our study (Brandl *et al.* 2012). However, diarrhoea in the homozygous *ADAM17* mutant children studied started very early in life and, unlike for *ADAM17* mutant mice, the triggering event is unclear. Furthermore, it needs to be noted that multiple reasons, unrelated to *ADAM17* deficiency, exist for early-onset diarrhoea with resolution later in life, and that changes in gastrointestinal tract physiology and function during development, with particular regards to the composition of the intestinal microbiota, may account for or contribute to episodes of acute diarrhoea. Duodenal biopsies, collected from the affected boy and the affected girl at the age of six and three years, respectively, showed only mild inflammatory changes, and the presence of villous atrophy. In the study by Bandsma *et al.* (Bandsma *et al.* 2015), a duodenal biopsy of the affected child showed mild focal villous atrophy and crypt hyperplasia without intraepithelial lymphocytosis. These alterations are similar to those observed in the small intestine of mice with *tace^{ΔZn/ΔZn}* mutation (Peschon *et al.* 1998), and may be related at least in part to a maturation defect in the intestinal epithelium, however the cause of the intestinal problems in both affected siblings has not been satisfactorily resolved and requires more investigation.

ADAM17 deficiency in humans has important immunologic consequences on the systemic immune response. PMA induces protein kinase C activation, which, in

turn, activates ADAM17 (Xu *et al.* 2010). Bandsma *et al.* (Bandsma *et al.* 2015) reported that both CD4⁺ and CD8⁺ peripheral blood T cells from the affected child showed normal activation following stimulation with PMA, phytohaemagglutinin, or staphylococcal enterotoxin B. It has been observed that TACE inhibition promotes IL-1 β and IFN- γ production by PBMCs (Sharma *et al.* 2013). However, in our study, levels of LPS-induced production of IL-1 β and levels of PMA- or anti-CD3/CD28-induced production of IFN- γ by PBMCs isolated from the affected boy at 17 years of age were comparable to those of the unaffected mother and control subjects. Interestingly, in the same experiments, levels of LPS-induced production of IL-6 by PBMCs from the affected boy were higher than those of control subjects. Conversely, levels of LPS-, PMA-, and anti-CD3/CD28-induced soluble TNF- α produced by PBMCs isolated from the affected boy were substantially impaired compared to those of the unaffected mother and control subjects, and this is likely to be a consequence of reduced TACE activity due to ADAM17 mutation. Using intracellular staining and flow cytometry, Bandsma *et al.* (Bandsma *et al.* 2015) reported that peripheral blood T cells from the affected child in their study displayed up-regulated IFN- γ and down-regulated IL-2 and TNF- α . It is difficult to explain their result on TNF- α production, as ADAM17 is known to exert its TACE activity on transmembrane, rather than intracellular, TNF- α . Other molecules with TACE activity, such as ADAM9, ADAM10, and ADAM19, may contribute to the residual production of soluble TNF- α by PBMCs from humans with ADAM17 deficiency. Conditional knock-out mice lacking ADAM17 in myeloid cells are protected from TNF- α -mediated endotoxin shock (Horiuchi *et al.* 2007). On the other hand, the impaired production of soluble TNF- α might have contributed to the increased susceptibility of the affected boy to opportunistic infections of the skin, such as *otitis externa* from *S. aureus* and paronychia from *Candida*.

The affected boy in our study presented with a generalised pustular rash with flares of erythema and scaling. The skin of both affected siblings, in the first

years of life, was prone to infection with *S. aureus*. Also, in the study by Bandsma *et al.* (Bandsma *et al.* 2015) the affected child developed skin lesions, including pustular rash and erythema, and skin biopsies revealed parakeratosis, neutrophil infiltration, and spongiosis, with bacterial swabs of the skin being positive for *S. aureus* on multiple occasions. Keratinocytes from the affected boy in our study expressed ADAM10, which has substrates in common with ADAM17 (Le Gall *et al.* 2009). The desmosomal cadherin DSG2, expressed in the less differentiated layers of the epidermis and hair follicle, has been shown to be a substrate of both ADAM17 and ADAM10 (Bech-Serra *et al.* 2006). The up-regulation in DSG2 expression that we observed in skin and keratinocytes of the affected boy suggests that ADAM17-mediated regulation of DSG2 availability at intercellular junctions is important in the skin (Klessner *et al.* 2009).

Finally, both the affected siblings in our study showed cardiac abnormalities. In particular, the affected girl died from fulminant *Parvovirus B19*-associated myocarditis, and the affected boy had left ventricular dilatation, with end diastolic dimension of 60 mm. Interestingly, in the study by Bandsma *et al.* (Bandsma *et al.* 2015), the affected child developed systolic hypertension and had mild pulmonary valve stenosis and a small atrial septum defect. DSG2 is the predominant DSG expressed in cardiac myocytes, and DSG2 mutations are associated with arrhythmogenic and dilated cardiomyopathies (Syrris *et al.* 2007; Posch *et al.* 2008). The relationship between myocarditis and possible early-onset arrhythmogenic cardiomyopathy is well recognised (Delmar *et al.* 2010), and impaired DSG2 regulation by ADAM17 may be responsible for the cardiac manifestations in the affected siblings. In addition, the lack of TNF- α may have been, at least in part, responsible for the affected girl's death, given the cardioprotective role of TNF- α in acute myocarditis (Wada *et al.* 2001).

Relevant similarities with the clinical features present in the humans with ADAM17 deficiency have been reported in a patient with inherited homozygous loss-of-function missense mutation in *EGFR* gene. In particular, this patient

showed dry skin and alopecia during his first year of life. Subsequently, pustules and papules were present, similar to those described among the side effects of EGFR inhibitors (Lacouture 2006), associated with dysregulated differentiation of keratinocytes. The patient died at 2.5 years of age due to extensive skin and chest infections and electrolyte imbalance (Campbell *et al.* 2014).

Both our observations and the findings by Bandsma *et al.* (Bandsma *et al.* 2015) suggest that ADAM17 deficiency in humans is associated with down-regulated TNF- α signalling. Interestingly, the affected boy in our study had been previously treated with the anti-TNF- α agent adalimumab, and this did not induce any significant improvement, which would not be surprising given the impairment in TNF- α production. It has been reported that almost 5% IBD patients treated with anti-TNF- α agents develop psoriasiform skin lesions. These are characterised by infiltrates of IL-17A/IL-22-expressing Th17 cells and IFN- γ -expressing Th1 cells, and show a high response rate to the anti-IL-12/IL-23 monoclonal antibody ustekinumab (Tillack *et al.* 2014). The mechanism proposed to explain the paradoxical skin inflammation induced by anti-TNF- α agents involves the binding of the anti-TNF- α Fc region to the Fc γ RI of macrophages, and the subsequent activation and production of IL-23 (Niess *et al.* 2014). In genetic ADAM17 deficiency in humans, it does not seem likely that the skin lesions, which represent one of the predominant clinical features in all three cases described so far, derive directly from the impairment in TNF- α signalling. Nevertheless, the multiple effects of ADAM17 on the immune response may influence Th1 and Th17 cell development in specific tissues, including the skin.

Limitations of the study

Both in our study and in that of Bandsma *et al.* (Bandsma *et al.* 2015), patients showed features compatible with functional ablation of ADAM17, however we have not formally proved that the predicted truncated version of ADAM17 is expressed in humans with genetic ADAM17 deficiency, and whether residual or

truncated forms of ADAM17 are non-functional. It needs to be noted that mutations at different sites on the *ADAM17* gene produce specific alterations in ADAM17 protein. Therefore, comparing transgenic mice that express no ADAM17 with patients that may have some residual and potentially active protein may prove to be misleading and requires caution. Nevertheless, there are similarities between the phenotypes of *tace* ^{Δ Zn/ Δ Zn} mutant mice and humans with genetic ADAM17 deficiency. *ADAM17*^{ex/ex} mutant mice, which have a marked reduction, but not a complete deficiency, of ADAM17 also present with skin and hair alterations (Chalaris *et al.* 2010).

One of the main limitations in our study was the availability of a small number of samples to analyse, which made it difficult to draw definitive conclusions regarding the consequences of *ADAM17* deletion. Unfortunately, the affected girl died before the start of our study, therefore it was not possible to perform the same investigations as in the affected boy, and it was also not possible to study matched control subjects due to the age of the affected boy. An example of the consequences of the limited number of samples and experiments is our observation that PBMCs from the unaffected mother showed down-regulated ADAM17 expression. This may reflect her being heterozygous carrier of the *ADAM17* mutation, however it is not possible to exclude that this is the effect of interindividual variability, and it has to be noted that this is the result of a single experiment. Nevertheless, the production of soluble TNF- α by PBMCs from the unaffected mother was comparable to control subjects.

Future studies

Unfortunately, due to the limited availability of samples from the affected boy and the unaffected relatives, we could only perform basic assays on cytokine release by PBMCs from the affected boy and the unaffected mother. In a future study on newly identified humans with genetic ADAM17 deficiency, it will be important to study the effects of *ADAM17* mutation on other important substrates, including

EGF, TGF- α , amphiregulin, IL-6R and L-selectin. Furthermore, it will be interesting to characterise the type of immune response driving the onset of cutaneous lesions, with particular focus on Th1 and Th17 cells and cytokines.

In parallel to impaired soluble TNF- α release by immune cells, ADAM17 deficiency could be associated to up-regulated transmembrane TNF- α expression due to defective cleavage. Transmembrane TNF- α accumulation, in turn, would have important consequences on intestinal inflammation (Mitoma *et al.* 2005). Despite being possible to assess the expression of the transmembrane form of TNF- α by flow cytometry using a commercially available antibody specific for transmembrane TNF- α , in the present study we could not perform this experiment due to the limited availability of samples. Hence, in a future study on newly identified humans with genetic ADAM17 deficiency, it will be important not only to confirm the down-regulation of intracellular TNF- α reported by Bandsma *et al.* (Bandsma *et al.* 2015), but also to explore the expression of transmembrane TNF- α on immune cells and the consequences on the systemic and mucosal immune response.

Inactive Rhomboid (iRhom)1 and iRhom2 are catalytically inactive intracellular serine proteases expressed in the endoplasmic reticulum that have emerged as important upstream regulators of ADAM17 (Lemberg *et al.* 2007; McIlwain *et al.* 2012). In particular, iRhom2, which is preferentially expressed in immune cells, promotes furin-mediated maturation and trafficking of ADAM17 to the cell membrane (Adrain *et al.* 2012). Moreover, iRhom2 has been shown to be the main regulator of ADAM17 in the skin (Brooke *et al.* 2014). Both *iRhom2*^{-/-} mice, which display defective TNF- α signalling, and *iRhom1*^{-/-} mice do not show any developmental abnormalities (Issuree *et al.* 2013; Li *et al.* 2015). Interestingly, *iRhom2*^{-/-} mice are protected from experimental arthritis, similarly to mice lacking ADAM17 in myeloid cells or TNF- α -deficient mice (Issuree *et al.* 2013). Conversely, *iRhom1/2* double knock-out mice display a severely impaired

survival rate and multi-organ abnormalities which resemble those of *ADAM17*^{-/-} and *EGFR*^{-/-} mice (Li *et al.* 2015). On the other hand, it has been shown that deletions in the cytoplasmic domain of iRhom1 and iRhom2 enhance ADAM17 activity *in vitro* in fibrosarcoma cells, thereby stimulating TNFR shedding (Maney *et al.* 2015). Furthermore, it has been reported that iRhom2 expression is up-regulated by TNF- α signalling (Adrain *et al.* 2012). In a future study on newly identified humans with genetic ADAM17 deficiency, it will be interesting to study whether there is any consequence on the expression of iRhom1 and iRhom2, such as a compensatory up-regulation, or a down-regulation consequent to impaired TNF- α signalling. More generally, it would be particularly intriguing to investigate iRhom1 and iRhom2 expression in IBD inflamed mucosa, where ADAM17 activity has been reported to be up-regulated (Monteleone *et al.* 2012), and to be a possible factor contributing to the excessive production of soluble TNF- α .

Chapter 7: General discussion and future directions

In this Thesis, we investigated aspects of disease pathogenesis and responsiveness to treatment in chronic intestinal inflammation using human tissue and various model systems. Experiments performed using human intestinal tissue, although not formally validated and not allowing mechanistic evaluations, may represent an informative tool to study the mucosal immune response in IBD.

Advances in the pathogenesis of human intestinal inflammation

Our results on the role of IL-17 and IL-13 in CD intestinal fibrosis, and of IL-13 in IBD inflamed mucosa, overall, suggest a pro-fibrogenic action of IL-17A in CD intestinal fibrosis. Moreover, our observations point towards the absence of a role for IL-17E and IL-13 in CD intestinal fibrosis, and suggest that IL-13 does not play an important role in IBD intestinal inflammation. A substantial proportion of our data derives from descriptive, cross-sectional observations and experiments. When evaluating the relevance of a certain pathway in IBD, results which report the up-regulation or down-regulation of specific molecules in the intestinal mucosa of CD and UC patients have often been used as supportive evidence, in addition to data obtained in experimental models of intestinal inflammation. Nevertheless, the rationale underlying the first application of anti-TNF- α agents in CD was based on the observation of up-regulated concentrations of TNF- α in the intestinal mucosa, stool and serum of patients compared to control subjects (Kaser 2014). We also performed *in vitro* functional experiments on the role of IL-17 and IL-13 in IBD. For this purpose, we used cells isolated from CD and UC patients instead of cell lines derived from CRC, and our results were in agreement with our descriptive observations, supporting the biologic plausibility of the pro-fibrogenic role of IL-17A in CD, the absence of effects of IL-17E in CD intestinal fibrosis, and the lack of involvement of IL-13 in UC inflammation.

Investigations on the pathogenesis of IBD have largely involved the use of animal models of intestinal inflammation. One of the main advantages of this approach is the possibility to perform mechanistic *in vivo* experiments, and to obtain direct information on the immunologic processes underlying the onset of intestinal lesions. Nevertheless, several murine models of colitis, including DSS-induced, oxazolone-induced and TNBS-induced colitis, differ considerably from IBD, as they are triggered by the administration of a single, specific chemical agent to animals with a wild-type genetic background. While disease mechanisms underlying several experimental models of colitis have been dissected, animal models have shown limited ability to predict the efficacy of novel molecules for the treatment of intestinal inflammation, and numerous biologic agents that were successful in improving murine colitis have not been effective when subsequently tested in clinical trials in IBD (MacDonald 2010; Chang *et al.* 2014).

In addition to animal models, *in vitro* experiments, and observations using human tissue, the critical analysis of data from clinical trials – especially those with immunotherapies – is an important method to evaluate the relevance of a certain pathway or molecule in IBD (Auer *et al.* 2014; Kaser 2014). In particular, our observations on the absence of a role for IL-13 in the overall population of UC patients are in keeping with the results of the two phase IIa clinical trials with monoclonal antibodies against IL-13, anrukinzumab andtralokinumab, in active UC (Reinisch *et al.* 2015; Danese *et al.* 2015; Tilg *et al.* 2015). Treatment with anrukinzumab did not result in any significant change in faecal calprotectin at week 14 compared to baseline in UC, and at the highest dose an increase in this biomarker of intestinal inflammation has been observed, reflecting disease deterioration (Reinisch *et al.* 2015). Despite showing a significant effect in inducing clinical remission (18% in the tralokinumab group compared to 6% in the placebo group), tralokinumab has been ineffective in achieving the primary endpoint, which was defined as clinical response at week 8, in moderate-to-severe UC (Danese *et al.* 2015).

Another indirect indication that intestinal inflammation in the overall population of UC patients is not driven by IL-13 is represented by the results of two randomised clinical trials on the effects of the nematode *T. suis* in active UC and CD, respectively (Summers *et al.* 2005; Sandborn *et al.* 2013a). The study performed in UC showed a significantly higher clinical efficacy of *T. suis* compared to placebo (Summers *et al.* 2005). Although the quality of these studies was not sufficient to draw any conclusion regarding the efficacy of *T. suis* in IBD (Garg *et al.* 2014), this treatment, which should by definition trigger a Th2 mucosal immune response, was not associated with significantly more adverse events compared to placebo.

The anti-IL-17A monoclonal antibody secukinumab has been ineffective in a clinical trial in active CD (Hueber *et al.* 2012). This is not in disagreement with our results on the involvement of IL-17A in CD intestinal fibrosis, since patients with strictures causing obstructive symptoms were excluded from the clinical trial with secukinumab (Hueber *et al.* 2012). While ours and other groups' results suggest that IL-17A is indeed involved in CD intestinal fibrosis, they need to be validated with *in situ* analyses and on larger numbers of patients before this cytokine can be considered as a promising therapeutic target in fibrostenosing CD.

Finally, we cannot exclude that intestinal inflammation in humans with ADAM17 deficiency may be related to their genetic mutation. This would highlight the heterogeneity of the possible mechanisms underlying intestinal inflammation, which would therefore develop regardless of the impaired ability to produce soluble TNF- α . Due to its TACE activity, ADAM17 is an attractive target for the treatment of chronic inflammatory diseases characterised by excessive TNF- α production (Arribas *et al.* 2009; Saftig *et al.* 2011), including IBD, psoriasis and rheumatoid arthritis. However, ADAM17 has a multitude of different substrates, including molecules playing an important role in tissue development and maturation, epithelial barrier function, and immune response, and this is reflected

by the alterations affecting the intestine, eyes, skin and hair observed in mice with ADAM17 deficiency. Also humans with genetic ADAM17 deficiency develop lesions involving the intestine, the skin, and the cardiovascular system. These aspects need to be carefully taken into account when considering ADAM17 as a therapeutic target. Finally, the long-term survival rate in humans with ADAM17 deficiency is currently unknown, and further studies are needed to establish whether short-term inhibition of the ADAM17 pathway has similar effects to those observed in homozygous ADAM17 mutations.

Biomarker development in IBD

In our study on the proteolytic degradation of anti-TNF- α agents and its correlation with response to biologic treatment in IBD, we initially performed functional *in vitro* experiments that demonstrated the biologic plausibility of this mechanism in the context of IBD inflamed mucosa. Subsequently, we studied whether we could detect signs of proteolytic degradation in the mucosa and in the serum of IBD patients, and we observed that there was overall correlation between response to anti-TNF- α therapy and serum levels of MMP-3-/MMP-12-cleaved endogenous IgG and anti-hinge autoantibodies.

In addition to their significance towards gaining a better understanding of the immunologic mechanisms underlying intestinal lesions and responsiveness to treatment in IBD, our results are potentially relevant for disease biomarker development in this condition. In particular, as it is often the case when performing studies on human tissues, the most important feature emerging from our findings is the considerable degree of heterogeneity between different patients in IBD. Our findings on levels of IL-17A and IL-13 in IBD intestinal tissue show that there is a high degree of heterogeneity in mucosal cytokine expression between different patients. This is consistent with previous studies, which showed that IL-13 transcripts are not always detectable in samples from UC inflamed mucosa (Kawashima *et al.* 2011), and that IFN- γ and IL-17A expression

in IBD inflamed mucosa is variable across different patients (Kobayashi *et al.* 2008). We observed that there is also a high level of heterogeneity in MMP mucosal expression profile in different IBD patients, and that among IBD patients there are substantial differences in serum levels of MMP-3-/MMP-12-cleaved endogenous IgG and anti-hinge autoantibodies.

The high degree of immunologic heterogeneity present in IBD may have important diagnostic and therapeutic implications. Both IL-13 and IL-17A blockade have been ineffective in unselected groups of active CD and UC patients. The lack of efficacy of anti-IL-13 and anti-IL17A antibodies in IBD may not be due to the absence of involvement of these cytokines in the majority of patients, but rather to the fact that they may play a pro-inflammatory or pro-fibrogenic role only in a specific subset of patients. Furthermore, even in the case that IL-17A may play a role in the majority of patients with fibrostenosing CD, it is conceivable that its neutralisation would be effective only in the early phase of stricture development. Hence, the development of a biomarker capable to predict the onset of intestinal fibrosis, which is currently not available (Rieder *et al.* 2014), would be important for patient stratification and consequent therapeutic management in CD.

A similar approach has been developed and successfully applied in asthma. Based on the molecular mechanisms and the main cytokines involved, asthmatic patients have stratified in different “endotypes”, and response to specific biologic treatments varies accordingly (Kau *et al.* 2014; Agusti *et al.* 2016). In particular, it has been shown that therapies targeting IL-4, IL-5 and IL-13 are highly effective in patients with “type 2 high” asthma (Fajt *et al.* 2015). Furthermore, treatment with the anti-IL-13 monoclonal antibody lebrikizumab in asthma is more effective when patients are stratified according to molecular biomarkers, such as serum levels of periostin (Corren *et al.* 2011; Durham *et al.* 2016; Izuhara *et al.* 2016).

It has been shown that the humanised anti- β_7 integrin monoclonal antibody etrolizumab, that selectively binds the heterodimeric $\alpha_4\beta_7$ and $\alpha_E\beta_7$ integrins, is significantly more effective than placebo in inducing clinical remission in moderate to severe active UC (Vermeire *et al.* 2014). In UC patients treated with etrolizumab, high colonic expression of granzyme A and α_E integrin is associated to significantly higher rates of clinical remission and mucosal healing (Tew *et al.* 2016). Regarding anti-TNF- α therapy, it has been shown that CD patients with higher levels of transmembrane TNF- α^+ cells in the colon respond better to adalimumab than patients with low numbers of transmembrane TNF- α^+ cells in the colonic mucosa (Atreya *et al.* 2014). Interestingly, a subgroup analysis revealed that, differently from placebo, secukinumab induced a significant decrease in faecal calprotectin in CD patients with a specific polymorphism (rs4263839) in the *TNF-like ligand 1A (TL1A)* gene, which plays a role in Th17 differentiation (Hueber *et al.* 2012). Further clinical trials on IL-17A and IL-13 blockade in CD and UC, designed using strategies of patient stratification based on data from ours and future immunological studies, may prove to be more successful than unselective blockade of these cytokines in IBD.

Our results may contribute to clarifying the mechanisms underlying primary non-response to TNF- α neutralising agents, especially in severely active IBD patients who are likely to have a high mucosal proteolytic activity and therefore may benefit from a higher anti-TNF- α loading dose (Gibson *et al.* 2015). It has been reported in a pharmacokinetic study that UC patients have a faster clearance of the anti-IL-13 monoclonal antibody anrukinzumab compared to control subjects and patients with asthma (Hua *et al.* 2015). It will be interesting to investigate whether this derives from general metabolic differences, or whether proteolytic cleavage may influence half-life and effectiveness of monoclonal antibodies against IL-13 and IL-17A in IBD. Finally, our preliminary observations that serum levels of cleaved IgG and anti-hinge autoantibodies against cleaved IgG are particularly high in a subgroup of IBD patients who do not respond to anti-TNF- α agents may form the basis for subsequent, prospective studies, with the aim to

identify accurate serum biomarkers of responsiveness to biologic therapy, and ultimately to develop effective strategies of patient stratification in IBD.

In conclusion, we believe that results from ours and other studies underline the complexity and the high degree of disease heterogeneity in human intestinal inflammation. Future studies should aim to achieve a better definition of the different immunologic components involved in individual IBD patients, which is necessary in order to develop a pathway-based approach to the treatment of IBD (Bamias *et al.* 2016). This will involve the development of reliable biomarkers and predictors of disease progression, and the formulation of stratification strategies and treatment algorithms that will form the basis for personalised, precision medicine in IBD.

Chapter 8: References

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